

**MICROBIOLOGICAL ASPECTS OF AN OYSTER  
(*Crassostrea gigas*) HATCHERY, WITH SPECIAL  
REFERENCE TO LARVAL MORTALITIES.**

by

*Tom*

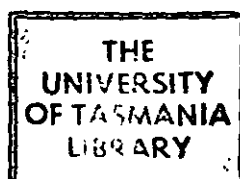
T.E. Lewis B.Sc (Hons.), Tas.

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This thesis contains no material which has been accepted for the award of any other degree or diploma in any University, and to the best of my knowledge contains no paraphrase of material previously published or written by any other person except where due reference is made in the text of this thesis.

A handwritten signature in black ink, appearing to read 'Tom Lewis', with a stylized, cursive script.

Tom Lewis  
University of Tasmania  
HOBART  
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## ABSTRACT

Investigations<sup>O</sup> of the cause/s of larval mortalities at a commercial Pacific oyster (*Crassostrea gigas*) hatchery in Tasmania, Australia, were carried out during successive production seasons. Epidemiological evidence suggested that cultures of microalgae, harvested semi-continuously as food for larval oysters (*Crassostrea gigas*) were the major vectors for transmission of bacterial disease. There was no evidence that bacteria associated with intake seawater, fertilised eggs or hatchery fomites (biofilmed surfaces) caused larval losses, or that non-bacteriological factors (eg. nutrition, gene pool, heavy metal contamination) were responsible for larval mortalities. All microalgal clones (*Isochrysis* sp. clone T. Iso., *Chroomonas salina* Butcher 3C, *Thalassiosira pseudonana* Hasle et Heimdal 3H, *Chaetoceros calcitrans* Paulsen, *Dunaliella salina* Butcher, *Pavlova* (*Monochrysis*) *lutheri* Droop, *Tetraselmis suecica* Butcher) used as food for larvae, were implicated in the transmission of disease.

It was apparent that mixed, rather than single, bacterial biotypes were responsible for disease. Bacteria cultured from moribund larvae rarely included presumptive *Vibrio* or *Flavobacterium* / *Cytophaga* spp., and never red-pigmented *Pseudomonas* spp.

When microalgal cultures containing more than  $\log_{10} 6.3$  culturable bacteria  $\text{mL}^{-1}$  were fed to larvae, the larval growth rate was reduced within 2-3 days. Small (15-25%), major (25-90%) or total (> 90%) losses occurred within 1-5 days. If no further cultures of microalgae exceeding the bacterial threshold were fed, surviving animals grew satisfactorily to the ready-to-set stage. If further cultures exceeding the bacterial threshold

level were fed further small, major or total ( $> 90\%$ ) losses were experienced within 1-7 days.

During the course of the study, techniques for the disinfection of microalgal growth medium were altered from pasteurisation to  $0.2\ \mu\text{m}$  membrane-filtration technology. This resulted in the mean levels of culturable bacteria in the growth media decreasing from  $\log_{10} 4.1\ \text{mL}^{-1}$  to  $\log_{10} 1.5\ 100\ \text{mL}^{-1}$ .

Scanning electron microscopic examination revealed a decrease in the proportion of microalgal cells colonised by bacteria when microalgal growth medium disinfection techniques changed from pasteurisation-cooling to membrane-filtration.

The proportion of microalgal cultures (at harvest) containing  $> \log_{10} 6.3\ \text{bacteria mL}^{-1}$  decreased from 55.6% (using pasteurised-cooled seawater as microalgal growth medium), to 20.6% and 10.9% in two successive production seasons during which  $0.2\ \mu\text{m}$  membrane-filtered seawater was used as microalgae growth medium. The yield of ready-to-set larvae rose during the same seasons from 1.3% to 3.2% to 4.6% of fertilised eggs.

## ABBREVIATIONS

Abbreviations commonly used in the text are listed below:

T. Iso	<i>Isochrysis</i> sp. (Tahitian clone)
3C	<i>Chroomonas salina</i>
3H	<i>Thalassiosira pseudonana</i>
C. cal.	<i>Chaetoceros calcitrans</i>
Dun.	<i>Dunaliella tertiolecta</i>
Pav.	<i>Pavlova lutheri</i>
Tet.	<i>Tetraselmis suecica</i>
DOM	dissolved organic matter
POM	particulate organic matter
SEM	scanning electron microscop(y/ic)
s	small (15-25%) loss of larvae
m	major (25-90%) loss of larvae
t	total (> 90%) loss of larvae
r, r-t-s	ready-to-set larvae
d	day
sd	standard deviation
PC	pasteurised/cooled
MF	0.2 $\mu$ m membrane-filtered
ppm	parts-per-million (w/v)
SWAV	Seawater agar with vitamins
TCBS	Thiosulphate-Citrate-Bile salts-Sucrose agar

## INTRODUCTION

The factors which limit the survival and growth of marine molluscan larvae in their natural environment include the abundance of food, settlement surfaces and predators, and fluctuations in temperature, salinity and turbulence. These factors are of much less importance in the controlled environment of modern shellfish hatcheries. Such controlled conditions are also favourable to the proliferation of marine bacteria. The importance of the role of bacteria in determining larval survival rates increases markedly, especially in hatcheries in which intensive (high density) culture techniques are employed. In some instances bacterial disease is the major limiting factor of bivalve larval development (e.g. Brown 1973, Brown and Russo 1979, Blogoslawski *et al.* 1978, 1980).

The association between bacteria and mortalities of laboratory reared marine mollusc larvae has been well established (Walne 1956, Guillard 1959, Tubiash *et al.* 1965, Murchelano *et al.* 1975). However, it has only been since the development of commercial hatchery ventures that bacterial diseases of mollusc larvae have become economically relevant. Commercial production of many species of marine mollusc has been achieved during the past decade. Due to the technology involved in such operations, slight variations in yield can determine the success or failure of the companies involved. Many marine mollusc hatcheries have failed, due to inadequate precautions against larval disease.

There are four major sources of bacteria in most shellfish hatchery operations: the intake seawater used as larval culture medium; the stocks of microalgae (or other feed stocks) used to feed the larvae; the fomites (e.g. the surfaces of pipes, sieves and culture tanks); and the broodstock used for spawning. Unless the degree of bacteriological contamination of each of these areas is monitored and controlled where necessary, bacterial

disease, caused by either specific pathogens or total bacterial numbers, can severely limit production.

During the 1983/84 production season, the commercial Pacific oyster (*Crassostrea gigas*) hatchery at Bicheno, Tasmania suffered serious losses of larvae. It was the aim of this study to identify the factors associated with the larval mortalities and to put in place microbiological procedures to prevent or minimise any further losses.

## LITERATURE REVIEW

### A. Factors Influencing the Abundance and Distribution of Planktonic Marine Bacteria.

For the purposes of this review, it is regarded that bacteria suspended in the water column (planktonic) exist either in a free-living state or are associated with particulate matter, detritus or other organisms.

#### 1.0 Influence of Nutrient Availability on Bacterial Activity.

The most significant determinants of the distribution of bacteria in the water column appears to be the concentrations of particulate and dissolved organic matter (POM and DOM respectively) (Millis 1981, Kogure *et al.* 1980, Fukami *et al.* 1983b). Temperature and hydrostatic pressure have also been shown to have some influence (Jannasch *et al.* 1971, Morita 1972, Morita 1974). Bacterial numbers and activity are generally higher in upper waters and coastal environments, reflecting higher temperatures (Morita 1974) and higher concentrations of organic nutrients resulting from land run-off and upwelling (Carlucci 1974, Ishida and Kadota 1974).

Estimates of the number of bacteria present in the water column in marine environments vary greatly, with recorded levels largely dependent on the nutrients available in the environment and the method by which the cells are counted. The advent of direct microscopic methods for counting planktonic bacteria (e.g. Hobbie *et al.* 1977) led to the realisation that conventional techniques using solid growth media estimated only a proportion of the total bacterial population. The technique of epifluorescence microscopy has further aided studies of the total bacterial numbers and biomass in aquatic environments (Taga and Matsuda 1974, Watson *et al.* 1977, Krambeck *et al.* 1981). However, direct observation of

fixed bacterial cells gives little indication of the taxonomic diversity of the bacterial population being studied, nor does it allow any study of ecological relationships between bacteria and other members of the plankton.

Estimates of the relative proportion of bacteria either free-living or associated with particles in the water column vary considerably, and are also largely dependent on the techniques by which the counts are obtained. Taga and Matsuda (1974) reported that, in surface oceanic seawater, viable counts indicated approximately 100 bacteria  $\text{mL}^{-1}$  attached to plankton, with only 1  $\text{mL}^{-1}$  free-living. However, direct counts revealed that free cells ( $10^3$ - $10^4$   $\text{mL}^{-1}$ ) formed the majority of the population, with the number of attached cells being in the order of  $10^2$ - $10^3$   $\text{mL}^{-1}$ . Similar results for direct counts of free-living and attached bacteria in coastal waters have been reported (Kogure *et al.* 1980, Cammen and Walker 1982, Boak and Goulder 1983, Fukami *et al.* 1983b).

Increased bacterial numbers, obtained by both direct microscopic and viable culture methods, are usually observed in eutrophic waters and in summer, when DOM levels are at their annual peak (Seki 1971, Taga 1974 cited in Fukami *et al.* 1983a). Suspended particulate organic matter (POM) has also been shown as a source of nutrients for marine bacterioplankton (Cammen and Walker 1982, Fukami *et al.* 1983b). Both these studies showed a correlation between the number of bacteria in the water column and the concentration of POM. However, in the latter study viable counts showed a greater correlation with the concentration of POM than did direct counts, indicating a variation in the nutritional requirements between free-living and attached bacteria.



### 1.1 Responses by Planktonic Bacteria to Low Nutrient Levels.

Generally, the amount of DOM in oceanic seawater is very low, seldom exceeding  $1\text{--}6\ \mu\text{g C.mL}^{-1}$  (Wagner 1969, Kushner 1978). Marine bacteria have developed a number of mechanisms to allow them to survive in such nutrient poor conditions. Oligotrophic bacteria, defined as those bacteria capable of growth on media containing  $1\text{--}15\ \mu\text{g C.mL}^{-1}$  (Kuznetsov *et al.* 1979, Ishida and Kadota 1981), are able to maintain normal metabolic activities in environments containing low substrate levels (Hirsch 1979) and may not respond to increases in nutrient availability when they do occur. Oligotrophic bacteria appear to have a low specificity, coupled with a high affinity, for organic nutrients, thus allowing them to make use of a wide variety of compounds (Poindexter 1981). Copiotrophic bacteria (Poindexter 1981) require relatively high levels of organic carbon for growth, and, in aquatic environments, are frequently outnumbered by their oligotrophic counterparts (Yanagita *et al.* 1978). Copiotrophic bacteria cannot function normally in oligotrophic environments and therefore need other characteristics to be able to survive periods of nutrient deprivation (i.e. starvation).

A common response by copiotrophic marine bacteria, when subjected to starvation conditions, is to form viable small (dwarf) forms which have a very low endogenous respiration rate and are thus able to survive extended periods of nutrient deprivation. A cogent argument for the ecological significance of dormant (dwarf) forms of bacteria during periods of nutrient limitation was put by Stevenson (1978). This author suggested that, due to the often large variations in nutrient availability in aquatic systems, most populations of bacteria would become extinct if they were not able to become dormant when conditions become unfavourable.

Novitzky and Morita (1976, 1977, 1978) described a psychrophilic marine *Vibrio* sp. able to survive severe nutrient limitations by

undergoing a dwarfing process, in which cell morphology changed from a rod of approximately 1  $\mu\text{m}$  (diameter) x 4  $\mu\text{m}$  (length) to a sphere approximately 0.4  $\mu\text{m}$  in diameter. Humphrey *et al.* (1983) also noted a dwarfing response to starvation conditions in several rod-shaped marine bacteria, although not in 3 coccoidal bacteria. These authors also found an increased affinity for surface colonisation by the starved bacteria. Kjelleberg *et al.* (1982) suggested that a nutrient poor surface causes a triggering effect which elicits the dwarfing process in some metabolically competent bacterial cells: the presence of any assimilable nutrient at a surface resulted in the inhibition and reversal of dwarfing until that nutrient was exhausted. It is apparent that metabolically competent cells possess enzymes specific for the dwarfing process and that dwarfing is an active process carried out by metabolically competent cells (Amy and Morita 1983, Humphrey *et al.* 1983).

Novitzky and Morita (1978) observed an increase in cell numbers (up to 400-fold) which coincided with the dwarfing of a marine *Vibrio* sp. when subjected to severe nutrient limitation. However, there was no significant increase in the biomass of the population during the dwarfing process, suggesting that cell division was supported by utilisation of cell reserves. Similar responses to starvation conditions have also been found in another marine *Vibrio* sp. (Dawson *et al.* 1981).

Although dwarf cells show an increased tolerance for nutrient poor conditions, the rapid increase in numbers at the onset of the dwarfing process does not always lead to a long term increase in the viable population of the bacteria. The viability of starving cells decreases steadily over time, showing half-lives in the order of 2-3 days (Dawson *et al.* 1981) to several weeks (Novitzky and Morita 1978, Tabor *et al.* 1981, Amy and Morita 1983, Amy *et al.* 1983). Extended periods of nutrient

deprivation will eventually lead to the total loss of a population of copiotrophic bacteria from an oligotrophic environment.

### 1.1.1 Abundance of Dwarf Bacterial Cells in Marine Environments.

Direct microscopic observation of bacterial cells collected from marine environments has led to the discovery that very small bacterial cells frequently outnumber normal-size cells in marine oceanic environments. Direct (microscopic) counts of very small bacteria, varying from  $10^2$  to  $10^7$  cells  $\text{mL}^{-1}$  have been reported from aquatic systems (Zimmerman 1977, Meyer-Reil 1978, Kogure *et al.* 1984). Qualitative studies (Oppenheimer 1952, Anderson and Heffernan 1965, Tabor *et al.* 1981, MacDonell and Hood 1982, Li and Dickie 1985) have also found significant numbers of viable and non-viable filterable bacteria in seawater (i.e. able to pass through either 0.45  $\mu\text{m}$  or 0.2  $\mu\text{m}$  pore size membrane filters; Table 1). These bacteria are thought to be either dwarf forms of usually normal sized bacteria, or oligotrophic ultramicrobacteria which have adapted to their environment by an irreversible decrease in cell size and which are metabolically active in nutrient poor conditions (Torrella and Morita 1981). The presence of dwarf- and ultramicro- bacteria may be an indication of the low nutrient status of the water.

The relative abundance of very small bacteria in seawater samples varies considerably. Tabor *et al.* (1981) found that bacteria able to pass through a 0.45  $\mu\text{m}$  filter comprised 0.5-77% of the total viable populations in twelve samples of seawater. The smallest percentages of filterable bacteria were located in coastal and estuarine samples. The estimates of the proportion of viable bacteria in filtered seawater samples were made using full strength media. It therefore appears that, in this case, viable filterable cells were dwarf forms of copiotrophic bacteria, rather than oligotrophic ultramicrobacteria as described by Torrella and Morita (1981). By

definition, oligotrophic bacteria would not have been able to grow on such rich culture media.

Scanning electron microscopic observation of seawater samples has revealed bacterial cells of  $\leq 0.4 \mu\text{m}$  (smallest diameter) in parallel surface and deep water samples (Tabor *et al.* 1981). Bacteria in deep water samples were, however, significantly smaller than cells from surface water, with the diameter of rod-shaped cells "notably reduced". Culturable bacteria comprised between 0.01-0.1% of the total (direct microscopic count) populations in the water samples. No values for direct counts of cells in the  $0.45 \mu\text{m}$  filtrate were given.

Although most dwarf cells show some evidence of metabolic activity it is likely that many of them are unable to respond to an increase in nutrient availability by growing and dividing (Dawson *et al.* 1981). Kogure *et al.* (1984), using a direct microscopic technique designed to differentiate between viable and non-viable dwarf cells in water samples, found that viable cells comprised about 10% of the total population, which ranged from  $10^5$  to  $10^7$  cells  $\text{mL}^{-1}$ . Plate-viable (culturable) counts were 1-2 orders of magnitude below the direct viable count. It seems that this technique will not detect viable (oligotrophic) ultramicrobacteria, as defined by Torrella and Morita (1981). Differentiation of viable and non-viable cells, using the method of Kogure *et al.* (1984), depends on viable dwarf bacteria expanding to "normal" size upon the addition of dilute nutrients. Ultramicrobacterial cells will not expand significantly after the addition of nutrients (Torrella and Morita 1981).

Table 1. Marine bacteria able to pass through membrane filters.

Habitat	Filter pore size ( $\mu\text{m}$ )	Bacteria Viable count	$\text{mL}^{-1}$ Direct count	Cell size range ( $\mu\text{m}$ )	Genera of bacteria	Reference
Estuarine	0.4	0-12	ND <sup>a</sup>	ND	ND	Oppenheimer 1952
Estuarine	0.2	ND	ND	< 0.2	<i>Vibrio</i> spp. (65%) <i>Aeromonas</i> spp. (13%) <i>Alcaligenes</i> spp. (9%) <i>Pseudomonas</i> spp. (9%) unknown (4%)	MacDonell and Hood 1982
Oceanic (surface)	0.2	1-30	ND	ND	<i>Vibrio</i> spp. <i>Spirillum</i> spp. <i>Flavobacterium</i> spp. <i>Cytophaga</i> spp. <i>Leucothrix</i> spp.	Anderson and Heffernan 1965
Oceanic (>1000m) <sup>b</sup>	0.45	0.01-1	ND	< 0.4	ND	Tabor <i>et al.</i> 1981
Oceanic (10-80m) <sup>b</sup>	0.2	+ <sup>c</sup>	1-10 <sup>5</sup>	ND	ND	Li and Dickie 1985

<sup>a</sup>: Not Determined;    <sup>b</sup>: depth below surface;    <sup>c</sup>: + = growth recorded

In the absence of inhibitors of cell division, Kogure *et al.* (1984) found that viable (cultured) counts of bacteria in seawater, to which dilute nutrients had been added, began to increase rapidly after 9-10 hours incubation at 20°C. This was due, in part, to active division of normal size viable cells. It is also possible that some dwarf cells recovered sufficiently during incubation, as evidenced by their increased cell size, and also started to divide.

## 2.0 Microalgae as a Source of Dissolved Organic Matter.

A great number of reports has been published concerning the release, by microalgal cells, of DOM, including amino acids, mono-saccharides, poly-alcohols, and volatile organic acids, as reviewed by Hellebust (1965) and Fogg (1977). Estimates of the amount of material released, as a proportion of total microalgal production are variable, ranging from 0-20% (Hellebust 1965, Thomas 1971, Ignatiades 1973) to approximately 75% (Choi 1972, Al-Hasan *et al.* 1975). Sharp (1977) concluded that many reported values of DOM production by microalgae are overestimated, due to errors in experimental design and interpretation of results. A value of 0-5% of total microalgal production being released as DOM was suggested, by Sharp (1977), as being more realistic.

This does not mean that release of DOM from healthy cells is not significant. Mague *et al.* (1980) concluded that release of DOM was a normal function of healthy cells and was closely related to photosynthetic rate. Studies on the relative rates of DOM release by microalgal cells have shown that populations in the lag, stationary and decline phases release more DOM than cells in the log phase of growth (Guillard and Wangersky 1958, Marker 1965, Jolley and Jones 1977, Larsson and Hagstrom 1979). Much of the maximal population phase material probably comes from lysing cells (Jones 1982).

## 2.1 Response by Bacteria to DOM Produced by Microalgae.

Bacteria and other microorganisms have long been known to play an essential role in marine ecosystems. While early workers first thought marine bacteria acted mainly as remineralisers (Zobell 1946) they are now believed to recycle large amounts of dissolved organic matter back into the food web (Azam *et al.* 1983).

In natural seawater, peaks in heterotrophic bacterial metabolic activity are localised occurrences, related to increases in nutrient availability in the water column (Sieburth 1976). The size of the bacterial population in the water column also generally varies in proportion to the density of microalgal populations present (Zobell 1946, Fuhrman *et al.* 1980, Millis 1981). These results, in conjunction with the finding of a strong relationship between primary production and release of organic substances (Wolter 1982), suggest that microalgal extracellular products are an important source of nutrients for planktonic bacteria.

### 2.1.1 Batch Culture Experiments and Observations of Natural Microalgal Blooms.

*In situ* transfer of organic material from healthy microalgae to surrounding bacteria has been demonstrated using  $^{14}\text{C}$ -labelled compounds (Nalewajko *et al.* 1976, Jolley and Jones 1977, Larsson and Hagstrom 1979, Bell and Sakshaug 1980, Fuhrman *et al.* 1980, Cole *et al.* 1982, Wolter 1982). Bell (1984) reported that utilisation of microalgal extracellular organic compounds is a general feature of metabolically active bacterial populations from natural waters.

It appears that the low level of recorded extracellular microalgal DOM is a result of the rapidity of its uptake by planktonic bacteria. Bell (1980) showed that the rate of metabolism of algal DOM by bacteria is

controlled by an enzyme-mediated transport system and that the rate of uptake of DOM is substrate- rather than transport-limited. This indicates that DOM excreted by microalgae is usually incorporated by bacteria almost immediately after it enters the water column. Similar reports have been made by Derenbach and Williams (1974) and Wolter (1982).

Some marine bacteria have been reported to exhibit positive chemotaxis towards environmentally significant concentrations of microalgal DOM and pure substrates known to be excreted by microalgae (Bell and Mitchell 1972, Torrella and Morita 1981, Kogure *et al.* 1982b)). Bell and Mitchell (1972) also reported that filtrates from older microalgal cultures elicited a greater chemotactic response by, and growth rates of, the test bacteria than filtrates from young cultures. Similar results, based on both direct microscopic and viable counts, were reported by Fukami *et al.* (1981a, 1983a,b). These findings support the idea of increasing production of DOM by microalgal cells in the lag or stationary growth phases, as described earlier.

The number of bacteria in close proximity to microalgal cells generally increases with the age of a microalgal bloom but is not correlated to the number of bacteria initially present in the water column (Fukami *et al.* 1981b, 1983a, Kogure *et al.* 1982a). The method used by these authors to differentiate "attached" and "free-living" bacterial cells involved separation of the free-living from the attached bacteria by means of filtration through 3 or 5  $\mu\text{m}$  disc filters. The shear forces exerted by the filtration process may have dislodged some or all of the reversibly attached cells from the surface (Dawson *et al.* 1981, Hermansson and Marshall 1985). Thus the estimates of "free-living" cells in these studies may be artificially high.

Although up to 50% of bacterial heterotrophic activity in natural waters is supported by microalgal extracellular products (Larsson and



Hagstrom 1979, 1982, Burney *et al.* 1982), it cannot be inferred that metabolic activity in all marine bacteria is stimulated by extracellular DOM from all marine microalgae. As described earlier, the range of compounds known to be released by microalgae is wide and well established. However, there is a paucity of information regarding bacterial utilisation of specific compounds extracted directly from microalgal cells. Berland *et al.* (1970). studied bacterial utilisation of a wide range of compounds, many of which were known to be released by microalgae. These authors concluded that different species of bacteria, even from the same genus, have widely different nutritional needs, with many requiring the presence of "growth factors" to allow specific compounds to be utilised. Thus the composition of the DOM pool released by a microalgal bloom would have a marked influence on the taxonomic diversity of the ensuing bacterial population. These results can be used to explain the reports by Fukami *et al.* (1981b, 1983a) and Kogure *et al.* (1982a), who found no apparent correlation between the taxonomic diversity of the bacteria associated with a microalgal population and that in the adjacent water column.

Such results strengthen the case of Bell and Mitchell (1972) for the existence of a "phycosphere" around all microalgal cells. The phycosphere was suggested to be the zone around a microalgal cell in which the extracellular DOM exerts some influence on the composition of the bacterioplankton.

Developing the concept of the phycosphere, Bell *et al.* (1974) showed extracellular DOM produced by the marine diatom *Skeletonema costatum* influenced two strains of marine bacteria very differently. The metabolic activity of a pseudomonad was stimulated in the presence of algal cells in the log phase, while that of a *Spirillum* sp. was inhibited by growing algal cells. Cell division of both species of bacteria was stimulated during

senescence of batch cultures of the alga. Very similar results have been found for a *Flavobacterium* sp. and *Pseudomonas* sp. respectively (Kogure *et al.* 1979, 1982b). These reports indicate that algal extracellular products may act to favour specific bacterial populations during algal blooms by means of selective stimulation or inhibition.

Inhibition of the attachment of bacterial cells to actively growing microalgal cells has been observed (Droop and Elston 1966, Kogure *et al.* 1982a, Bratbak and Thingstad 1985). Inhibition of this type is possibly caused by the production of low levels of antibiotics by some algal species. A range of marine microalgae has been shown to produce extracellular compounds which inhibit bacterial growth (Duff *et al.* 1966). Very little work has been performed in an attempt to characterise the inhibitory compounds (Table 2).

### 2.1.2 Continuous Culture Experiments.

Populations of bacteria associated with long term natural microalgal blooms, or with experimental continuous cultures of microalgae, show an increasing affinity for organic substrates produced by the microalgae over time (Bell 1983). When a mixed population of bacteria is kept in continuous culture, in the laboratory, with a single microalgal species, the bacteria undergo a clear process of adaptation: bacterial species showing a greater affinity for compounds released by the microalgae become dominant after several weeks (Bell and Mitchell 1972, Bell 1984). The period of time for adaptation to occur varies.

The diversity of bacterial populations maintained in continuous culture with microalgae increases with increasing stability of the system. Any perturbation of the system leads to transient decreases in bacterial diversity (Martin and Bianchi 1980). (This is a typical response to environmental perturbation by microbial populations in continuous culture

Table 2. Compounds of microalgal origin which inhibit bacterial growth.

Microalga	Compound	Reference
<i>Chlorella</i> sp.	"chlorellin"	Pratt <i>et al.</i> 1944
<i>Phaeocystis</i> sp.	acrylic acid	Sieburth 1961
phytoplankton	unidentified protein	Saz 1963
<i>Navicula delognei</i>	hexadecatetraenoic acid octadecatetraenoic acid hexadecatrienoic acid	Findlay and Patil 1984

systems; Harder *et al.* 1977). Kinetic studies of the uptake of  $^{14}\text{C}$ -labelled algal DOM partially support this hypothesis. Bell (1980) found that populations adapted to the DOM pool from one species of microalgae were not precluded from high-affinity uptake and metabolism of DOM from other algal sources. However, rates of uptake of the new DOM pool were shown to increase with time, indicating a period of adaptation, possibly linked to a short term lowering of taxonomic diversity.

### 3.0 Other Interactions Between Bacteria and Marine Microalgae.

#### 3.1 Bacterial Stimulation of Microalgal Growth.

Examples of stimulation of microalgal growth by bacteria are shown in Table 3. Such interactions are not easy to classify, as the degree of stimulation seems to depend on the culture conditions and the source of nutrients for the bacteria.

Jolley and Jones (1977) found that a *Flavobacterium* sp. and the diatom *Navicula muralis* both exhibited increased growth rates when grown together in a mixed culture than when cultured separately. Live cells of two strains of *Vibrio anguillarum* and one of *Escherichia coli* caused increases in the growth of up to 10 species of marine microalgae when mixed cultures were prepared on solid (agar based) growth media (Ukeles and Bishop 1975). As no stimulation of microalgal growth was recorded when liquid (artificial seawater based) growth media was used, it appears that stimulation was elicited through the release of growth stimulants by bacterial hydrolysis of the solid growth media.

Table 3. Bacteria which promote microalgal growth.

Microalga	Bacteria	Reference
<i>Chlorella</i> sp., <i>Nannochloris</i> sp., <i>Tetraselmis</i> sp., <i>Platymonas</i> sp., <i>Pyramimonas</i> sp., <i>Nitzschia</i> sp., <i>Dunaliella</i> sp., <i>Phaeodactylum</i> sp., <i>Stichococcus</i> sp..	<i>Vibrio anguillarum</i>	Ukeles and Bishop 1975
<i>Chlamydomonas</i> sp.	<i>Pseudomonas</i> sp. <i>Flavobacterium</i> sp.	Delucca and McCracken 1977
<i>Navicula muralis</i>	<i>Flavobacterium</i> sp.	Jolley and Jones 1977

Table 4. Bacterial pathogens of microalgae.

Microalgae species affected	Bacterial pathogen	Reference
<i>Chlorella</i> sp.	<i>Caulobacter vibrioides</i> (lysed)	Zavarzina 1964
<i>Chlamydomonas</i> sp.	<i>Cytophaga</i> sp.	Stewart and Brown 1969
<i>Skeletonema</i> sp., <i>Chlamydomonas</i> sp.,	<i>Pseudomonas</i> sp.	Mitchell 1971
<i>Thalassiosira</i> sp., <i>Chlamydomonas</i> sp., <i>Chaetoceros</i> sp., <i>Asterionella</i> sp., <i>Nitzschia</i> sp.	various	Berland <i>et al.</i> 1972
<i>Scenedesmus acutus</i>	Spirillum-like sp.	Schnepf <i>et al.</i> 1974
<i>Thalassiosira pseudonana</i>	<i>Pseudomonas</i> sp.	Baker and Herson 1978
<i>Chlorella</i> sp.	<i>Arthrobacter</i> sp.	Berger <i>et al.</i> 1979
<i>Chlamydomonas</i> sp.	<i>Pseudomonas-Flavobacterium- Xanthomonas</i> spp. (mixed)	Delucca and McCracken 1979
<i>Scenedesmus</i> sp.	various	Colwell and Speidel 1985

### 3.2 Bacterial Inhibition of Microalgal Growth.

Some species of bacteria have been found to inhibit the growth of microalgae (Table 4). Mechanisms by which inhibition is mediated are usually complex. Baker and Herson (1978) isolated a bacterium associated with the diatom *Thalassiosira pseudonana* which inhibited the growth of the diatom by a combination of nutrient competition and the production of an extracellular toxin, which was possibly a protein.

Delucca and McCracken (1979) reported a range of interactions between various microalgae and pure and mixed cultures of bacteria. These authors found that growth of a *Chlamydomonas* sp. was inhibited by mixed cultures of *Pseudomonas*, *Flavobacterium* and *Xanthomonas* spp. but was stimulated by pure cultures of the same *Pseudomonas* or *Flavobacterium* spp. Growth of the microalgae species examined was not affected by pure cultures of *Xanthomonas* sp.

The concentration of inhibitory compounds experimentally required to elicit inhibition is usually higher than would be found in natural ecosystems (Berland *et al.* 1972). However, laboratory cultures of microalgae can contain levels of bacteria far higher than may occur in open waters. (Murchelano and Brown 1969). In these cases bacterial inhibition of microalgal growth may become significant.

### 3.3 Bacterial/Microalgal Competition.

Some marine bacteria have the capacity to outcompete microalgae for a number of important nutrients, including phosphates (Rhee 1972, Currie and Kallf 1984) and nitrates (Parker *et al.* 1975). It appears the advantage enjoyed by the bacteria is due in part to their smaller size, which provides them with a much larger ratio of surface area:volume than the microalgal cells, thus allowing a more efficient uptake. In nutrient-limited

systems the heterotroph:autotroph biomass ratio increases, although an equilibrium between the bacteria and microalgal populations is nearly always reached, with both bacteria and microalgae co-existing (Bratbak and Thingstad 1985).

In most reports of bacteria-microalgae interactions, the bacterial biota associated with the microalgae appears to be controlled by both the relative concentrations of attractants and/or repellants/antibiotics in the phycosphere, and the relative sensitivities of different bacterial taxa to these chemicals. *In vitro* studies of the relationship/s between single or experimentally mixed populations of bacteria and microalgae only give limited insight to bacteria-microalgae interactions. The ecological significance of these laboratory studies is not, however, clear and requires further study, since *in vivo* many other selective factors will influence the relative abundance of microalgae and planktonic bacteria.

## **B. Bacterial Biofilms in Aquatic Environments.**

### **1.0 Mechanisms of Biofilm Formation.**

The development of microbial films on surfaces in aquatic environments is a well documented phenomenon (Zobell 1972). Early workers observed that bacteria grew preferentially on submerged surfaces, when compared to bacterial numbers in the adjacent water column. The adsorption and concentration of organic and inorganic nutrients, which occurs at solid/liquid interfaces was found to stimulate the growth of many types of the bacteria which adsorbed to surfaces (Zobell and Allen 1935, Heukelelian and Heller 1940, Zobell 1943, Fletcher 1980, Baier 1980). It is now evident that bacteria in biofilms outnumber planktonic bacteria in



natural freshwater (Geesey *et al.* 1978) and marine (Wood 1975) ecosystems.

Dahlback and Pedersen (1982) examined a marine biofilm, forming on glass and of which bacteria were the only living component. The authors estimated that 13% of the dry weight of the biofilm was protein. Stouthamer (1973) found the protein content of bacterial cells to be approximately 50% (w/w) dry weight. This indicates the presence of a large proportion of nonproteinaceous material in the biofilm, probably mostly polysaccharide (Costerton *et al.* 1981a).

Biofilms develop on submerged surfaces as a result of the sequence of processes described by Characklis and Cooksey (1983) and outlined below:

- i) Transport of organic and inorganic molecules and bacterial cells to the submerged surface.

Initially, organic and inorganic molecules and bacteria are brought close to the surface by external forces (e.g. water currents, Brownian motion). Transport of nutrients and non-motile bacteria to facilitate intimate contact with a surface is dependent upon currents, wave motion, capillary flow or attraction similar to that of colloidal substances to surfaces (Marshall and Bitton 1980).

Further attraction of motile bacteria may occur via positive chemotactic responses towards the increased nutrient concentration at the interface. Chemotactic responses by a marine bacterium towards organic molecules have been observed at concentrations as low as  $10^{-7}$  M (Torrella and Morita 1981). Concentrations of specific amino acids in near shore waters usually average  $10^{-7}$ -  $10^{-8}$  M (Wagner 1969) which indicates that the response thresholds reported by Torrella and Morita (1981) are ecologically significant. Motile aquatic bacteria are capable of chemotactic

responses towards the nutrient gradient established near solid/liquid interfaces (Young and Mitchell 1973, cited in Marshall and Bitton 1980).

ii) Adsorption of organic and inorganic molecules to the surface, resulting in a

"conditioned" surface.

Spontaneous adsorption of molecules to a surface from the water column is initiated immediately upon immersion of the surface (Baier 1980). The adsorbed material is largely organic and has a moderate negative charge (Neihof and Loeb 1972, Loeb and Neihof 1975 cited in Characklis and Cooksey 1983). The physicochemical character of a submerged solid is invariably masked by adsorbed inorganic and organic substances (Fletcher 1980), resulting in the formation of a "conditioned" surface. Characteristics which have been determined to influence the attachment of bacterial cells to surfaces, and which are often changed by conditioning molecules include pH, surface tension, ion exchange and electrostatic properties (Zobell 1972).

iii) Adhesion of bacterial cells to the conditioned surface.

One of the most important observations of the formation of biofilms in natural environments is that it does not occur without the presence of preadsorbed biological macromolecules (Baier 1980). The process of bacterial attachment to surfaces has been classified into three stages (Marshall *et al.* 1971, Floodgate 1972 cited in Kogure *et al.* 1982a):

a. Reversible sorption, whereby the cells are held to the surface by weak electrostatic and Van der Waals forces.

Initial sorption of bacterial cells to submerged surfaces usually occurs within a few hours of immersion (Marshall *et al.* 1971, Fletcher 1980).

Variations in the degree of adsorption may reflect differences in the electrokinetic potentials of both the bacterial cells and the adsorbant surface (Daniels 1980). Factors which influence adsorption of bacteria to surfaces are shown in Table 5.

b. Irreversible sorption, whereby the cells become firmly attached to the surface.

The mechanisms by which aquatic bacteria irreversibly attach to inert surfaces vary considerably and are mediated by the same environmental and biological processes as reversible sorption (Table 5). However, the usual means by which bacteria irreversibly attach to surfaces is by the production of insoluble extracellular polysaccharidic structures (Paerl 1975, Fletcher 1980, Characklis and Cooksey 1983, Pringle *et al.* 1983). These structures are generically classified as the glycocalyx (Costerton *et al.* 1981a).

✓  
a) Fletcher (1980) suggested that some marine bacteria are able to adsorb irreversibly to a submerged surface without first achieving irreversible sorption, indicating the instantaneous production of attachment structures. It would seem more likely that a very short period of irreversible sorption occurs in these situations, thus allowing the bacteria some contact with the surface to stimulate production of extracellular adhesive compounds.

c. Biological attachment, whereby irreversibly attached cells begin to actively divide and form microcolonies in response to appropriate nutrient availability.

Continued development of the biofilm results from bacterial multiplication and accumulation of nutrients and detritus from the water column (Costerton *et al.* 1981a). Biofilm density increases with increasing

Table 5. Factors which influence the adsorption of aquatic bacteria to submerged inert surfaces.<sup>a</sup>

.....Characteristic of.....		
Bacteria	Surface	Environment
species	roughness	nutrient status
strain	ionic form	temperature
culture medium	hydrophobicity	turbulence
growth phase	surface free-	pH
concentration	energy	duration of contact
		[inorganic salt] <sup>b</sup>
		[cation]

<sup>a</sup>: from Corpe (1970), Daniels (1980) and Fletcher (1977, 1980).

<sup>b</sup>: [A] = concentration of "A" in the water column.

water turbulence and increased nutrient availability (Characklis and Cooksey 1983).

Periodic detachment of portions of the biofilm occurs during all phases of the development of a biofilm. The rate of detachment is dependent on the physical attributes of the biofilm and the influence of external factors (e.g. turbulence, nutrient availability) on the film. Thick biofilms tend to detach more easily than thin films and the rate of detachment is greater in areas of high water movement (Characklis and Cooksey 1983) resulting in a high rate of nutrient and bacterial biomass turnover .

The range of bacterial taxa involved in the formation of a primary biofilm usually differs from that in the adjacent water column, but is influenced by the bacteria dispersed in the adjacent water column and their relative abundance (Characklis and Cooksey 1983). The bacterial population in the biofilm undergoes a constant succession as each new member of the biofilm modifies the physicochemical properties of the surface in some way to make it suitable for subsequent colonising organisms (Corpe 1970, 1974, Marshall *et al.* 1971, Marshall 1976, Dempsey 1981). Unless energy is put into the system, in the form of mechanical removal of the biofilm, the diversity of the organisms in the biofilm will keep increasing until limited by some environmental factor (e.g space, light, nutrient availability, accumulation of toxic metabolites) (Bott and Brock 1970).

Microscopic observation of the formation of a biofilm reveals a succession of the types of bacteria colonising the surface. Rod-shaped bacteria are generally the first to colonise submerged surfaces, with coccobacilli numbers increasing more slowly (Marshall *et al.* 1971, Fletcher 1980). This is likely to be due to the greater motility of the rod-shaped

bacteria. However, it may also indicate the existence of some other selection mechanism which favours the early sorption of some rod-shaped bacteria. It should be noted that the initial bacterial biota in a biofilm is not always displaced by subsequent colonisers (Corpe 1970, Marshall *et al.* 1971).

Succession of bacterial taxa on a surface occurs by similar processes to those which influenced selection during initial bacterial colonisation. Successive populations alter the surface environment in some way (usually resulting in a change of surface hydrophobicity) and thus render it suitable for subsequent colonising organisms (Fletcher 1980).

Selection of the taxa adsorbing to a surface may also be mediated by variations in nutritional requirements of different bacteria in the water adjacent to the submerged surface. Oligotrophic bacteria may not respond to the increased nutrient concentration at interfaces as they are adapted to low nutrient conditions (Kjelleberg *et al.* 1982). After initial colonisation of a surface by copiotrophs, Fletcher and Marshall (1982) suggested that oligotrophic bacteria may be the dominant group in the secondary colonisers. This could be due to a depletion of nutrients in the biofilm by the copiotrophs to a level at which oligotrophs have a competitive advantage. Why oligotrophic bacteria would need to adsorb to a surface is unclear, as by definition they are adapted to the low nutrient conditions in the water column.

Bacterial succession is not limited to the upper layers of a biofilm. As the depth of the film increases, oxygen and nutrient levels in the deeper parts of the film become depleted, due to limited diffusion of these compounds through the glycocalyx (Characklis and Cooksey 1983). Combined aerobic/anaerobic biofilms release considerably higher yields of organic products into the water column. The products are produced in the anaerobic portion of the biofilm and diffuse through the aerobic layer into

the water (Characklis and Cooksey 1983) with only a small fraction of them being consumed by organisms in the aerobic layer of the film. Conditions in the anaerobic layers of biofilms select for sulphate- and nitrate-reducing bacteria, resulting in increased production of sulphides and ammonia (Millis 1981).

### 1.1 Selective Advantages of Biofilm Formation.

Adhesion to a surface (i.e. biofilm formation) is a common mechanism for survival by marine bacteria in nutrient-poor conditions. Copiotrophic bacteria are generally enriched at these interfaces, depending on the degree of nutrient accumulation and specific nutrient requirements of the bacteria concerned (Marshall 1976, Kjelleberg *et al.* 1979, Dawson *et al.* 1981).

Not only are nutrients concentrated at the interface by colloidal mechanisms as already mentioned, but the glycocalyx itself acts to attract and entrap nutrients. The glycocalyx is polyanionic and acts like an ion exchange resin for further concentration of nutrients from the adjacent water column (Costerton *et al.* 1981b). Biofilms tend to be more adsorbant than clean surfaces and therefore can increase accumulation of detritus onto the surface (Zelver *et al.* 1982 cited in Characklis and Cooksey 1983). Increased rates of sedimentation, and thus nutrient sequestering, by the biofilm will lead to greater aerobic metabolic activity in the surface layer of the biofilm. Subsequent formation of an oxygen depleted environment towards the bottom of the biofilm will result from this increase in bacterial activity and thus allow the growth of anaerobic bacteria (Characklis and Cooksey 1983).

Other advantages conferred to aquatic bacteria growing within biofilms include: partial protection from rapid fluctuations in the concentrations of dissolved growth stimulating and/or inhibiting substances

in the adjacent water column (Costerton *et al.* 1981a); maintenance of position in a favourable environment (Marshall and Bitton 1980); accumulation of bacterial exo-enzymes and hydrolysates in the interstitial spaces of the glycocalyx, resulting in increased efficiency of utilisation of available nutrients (Zobell 1972).

A further advantage is that the biofilm acts as a reservoir of metabolically active cells which can break away and colonise other areas (i.e. *i.e.* metastasis) when conditions are favourable (Costerton *et al.* 1981a, Characklis and Cooksey 1983).

## 2.0 Potential Influences of Biofilms on Shellfish Hatchery Operations.

### 2.1 Positive Effects.

The increased metabolic activity of bacteria associated with biofilms, as described above, serves an important role in intensive aquaculture systems, especially in those systems in which much of the culture water is reused (recirculated). The potential for rapid increases of the concentrations of toxic metabolites in recirculated culture water is far greater than in 'flow-through' culture systems (Forteath 1990).<sup>a</sup>

Metabolic wastes of marine animals in dissolved or particulate form include ammonia ( $\text{NH}_3$ ), its oxidised derivatives nitrite ( $\text{NO}_2^-$ ) and nitrate ( $\text{NO}_3^-$ ) and various organic compounds with reduced nitrogenous moieties. These compounds can be toxic to aquatic organisms, although their respective toxicities depend on species, life stage and environmental factors (Illingworth *et al.* 1979, Forteath 1990). Of these compounds, ammonia is generally considered the most potentially toxic in aquaculture systems. Ammonia concentrations of  $>0.6\mu\text{mol}$  are considered highly deleterious for successful commercial aquaculture ventures (Huguenin and Colt 1989).<sup>a</sup>

Ammonia and urea, which is readily oxidised to form ammonia, can also enter aquatic systems from the decomposition of organic matter and from terrestrial runoff (Millis 1981). Urea and ammonia levels in near-shore waters are generally greater than in oceanic waters and often reach concentrations of approximately  $6\mu\text{mol}$  and  $0.1\mu\text{mol}$  respectively and constitute approximately 50-70% and 30-50% of the total N in these waters respectively (Remsen *et al.* 1974). Although these levels are not usually toxic to aquatic animals in natural systems, marine animals reared in

<sup>a</sup>: see Addendum, page 219.



intensive systems tend to be more sensitive to elevated levels of metabolites (Spotte 1970).

To counter the possibility of nitrogenous compounds reaching toxic concentrations in culture systems, many hatcheries use 'biological filters' to reduce the levels of these compounds in intake or recirculating water (Holliday 1985). In this case a biofilter is defined as a unit with a high internal surface area used for biological oxidation of organic compounds (Huguenin and Colt 1989).

Biological nitrification, and thus detoxification, of ammonia occurs as a two step process and is carried out under aerobic conditions by bacteria of the genera *Nitrosomonas*, *Nitrosococcus* and *Nitrobacter*. These bacteria (defined as nitrifying bacteria) readily colonise surfaces in natural systems with rates of nitrification on surfaces being far greater than those in the adjacent water column (Millis 1981). Nitrifying bacteria usually constitute the majority of bacterial populations in biological filtration systems (Spotte 1970).

Ammonia is first oxidised to nitrite by *Nitrosomonas* and/or *Nitrosococcus* (Forteath 1990). Nitrite is considered less toxic than ammonia but can still impact on some species. Nitrite concentrations of  $>10\mu\text{mol}$  have been shown to cause toxicity in salmonids (Mayo 1976). Nitrite is further oxidised to form nitrate by bacteria of the genus *Nitrobacter*. Nitrate is much less toxic than nitrite, with concentrations of  $<3\text{mmol}$  often being acceptable (Huguenin and Colt 1989).

Efficiency of nitrification in a specific biological filter is largely a function of surface area of the media in the filter and the time required for the water to pass through the filter bed (Liao and Mayo 1972, 1974). Biofilms act to accumulate compounds from the adjacent water column and as such are able to remove toxic metabolites from the water very efficiently. Mayo (1976) reports that biological filters can strip up to 98% of ammonia from a body of water under optimal flow rate (2-5 filter bed volumes  $\text{hour}^{-1}$ ) and temperature (20-25°C). However, such conditions are not practical for many commercial shellfish hatcheries, as typical water flow rates through filters (10-100 filter bed volumes  $\text{hour}^{-1}$ ; Holliday 1985) would not permit maximum removal of potentially toxic compounds.

## 2.2 Negative Effects.

The presence of biofilms on the internal surfaces of plumbing systems or culture vessels in a hatchery can have a number of negative effects on the operations of the hatchery. Thin biofilms can increase resistance to water flow through pipes (Characklis 1973) chiefly through increases in surface roughness (McCoy and Costerton 1982). This can lead to a significant increase in pumping costs. Biofilms can also decrease the efficiency of heat-exchangers, resulting in greater heating/cooling costs (Characklis and Cooksey 1983)

Metabolic processes of bacteria in the bottom layers of a biofilm can result in corrosion of the wetted surfaces of mild steel pipes (Characklis and Cooksey 1983). This problem has been largely eliminated with the use of poly-vinyl chloride instead of steel for the manufacture of water pipes. However, plastic pipes also deteriorate over time, and compounds can leach from the plastic into the water column. Such compounds include copper, zinc and cadmium complexes, which are often used as chemical stabilisers in PVC plumbing (Hardie Iplex P/L product catalogue 1988). These compounds can be toxic to marine animals, particularly larval and early juvenile stages.

Increased bacterial metabolic activity in the biofilms will result in increases in the release of bacterial metabolites into the water column (Zobell 1972), some of which may be toxic to the organisms under culture. Oxygen levels in the water may also be depleted although this problem can be overcome to a great extent by aerating the water further downstream (Mayo 1976).

Biofilms may harbour elevated levels of potentially pathogenic bacteria. For example, Garland *et al.* (1983) found high levels of *Vibrio* spp. on the wet surfaces of fouled pipes at a commercial oyster hatchery.

Sloughing of a biofilm results in sudden increases in the levels of bacteria in the water column which may cause disease in sensitive animals which are reared in the water (Elston 1984).

### C. Development of Crassostreid Oyster Larvae.

Crassostreid oyster larvae undergo a number of well defined stages during their development from freshly spawned gametes to newly settled juveniles. Knowledge of the basic embryology of the larvae is necessary in order to understand the descriptions of larvae given in this thesis. The following description of larval development (at 20-25°C) has been adapted from MacBride (1914) and Loosanoff and Davis (1963) and is summarised in Figure 1.

Soon after fertilisation, a polar body appears at the periphery of the egg membrane (Figure 1A). The egg undergoes its first division approximately 30 minutes after this (Figure 1B). As cleavage continues the prototrochal girdle (which is the precursor of the velum and contains the locomotory cilia), apical plate, digestive system, kidneys and genital organs all develop while the embryo is still encased in the egg membrane. When this stage of development has been attained the embryo bursts the egg membrane and enters its free-swimming life as a trochophore larva (Figure 1C).

The trochophore larva is only protected by a thin horny cuticle secreted by a shell gland situated in the region which will become the hinge in later stages. However, the larva quickly (usually within 24 hours) develops into the veliger stage (Figure 1D). This change takes place by means of the enlargement of the prototrochal girdle, to form the velum, and the secretion of the twin calcareous shells, the valves, from the shell gland. Young veliger larvae of oysters are more flattened than

trochophore larvae and have a characteristic "D" shape. In the next few days (at 20-25°C) the straight hinge becomes rounded, forming the umbone (Figure 1E).

During the next 2-3 weeks the developing veliger larva grows to a length (measured as maximum shell diameter) of approximately 300-350  $\mu\text{m}$ , it develops a foot with which it is able to achieve locomotion on solid surfaces (Figure 1F). At this stage the larva, which is now called a pediveliger, is fully competent to undergo settlement and metamorphosis.

Ready-to-set larvae enter a stage of alternate swimming and crawling during which they search for an appropriate settlement substratum. Once such a substratum is found a larva will resorb its velum, thus abandoning the planktonic stage. After a short (1-2 hour) period of crawling on the substratum the larva secretes a powerful cement by which it permanently attaches itself to the substratum and assumes a totally sessile life-style. The larva is now fully metamorphosed and is considered to be a juvenile (i.e sexually immature) oyster or spat.

#### **D. Beneficial Effects of Bacteria for Marine Mollusc Larvae.**

##### **1.0 Induction of Settlement and Metamorphosis.**

The life cycle of many bottom dwelling marine invertebrates begins with a planktonic larval stage (Crisp 1974). During this short stage of their life larvae must locate an area of substratum on which to settle (terminate their planktonic existence and assume a sessile or non-sessile sedentary life) and metamorphose (undergo morphological and physiological changes to become better suited to the benthos) (Scheltema 1974).

Although mollusc larvae are able to swim, by means of rapidly beating cilia attached to their vela, their locomotory power is not strong. In the wild, distribution of a cohort of larvae from the time of spawning to

Figure 1. Schematic representation of major stages of the development of larval oysters (adapted from MacBride 1914 and Loosanoff and Davis 1963).

A. Fertilised egg after release of first polar body.

Bar = 20  $\mu\text{m}$ .

B. Fertilised egg after first cell division.

Bar = 20  $\mu\text{m}$ .

C. Newly hatched trochophore larva.

A trochophore larva has an unprotected body as no shell is secreted until the veliger stage.

Bar = 20  $\mu\text{m}$ .

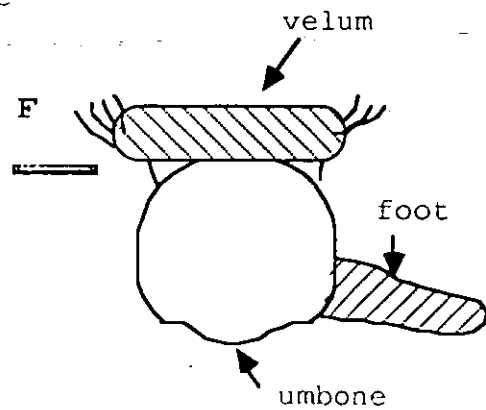
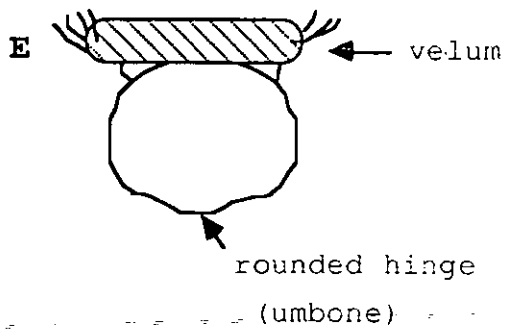
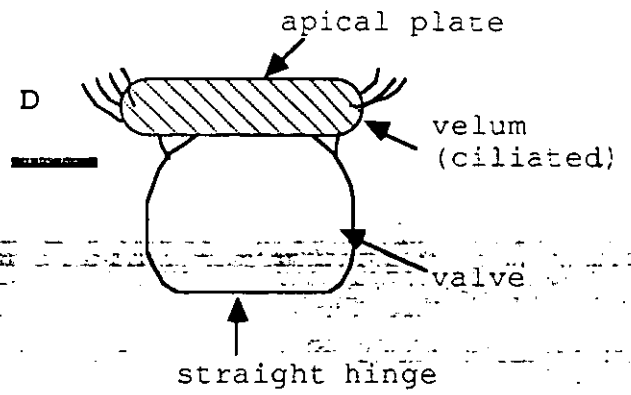
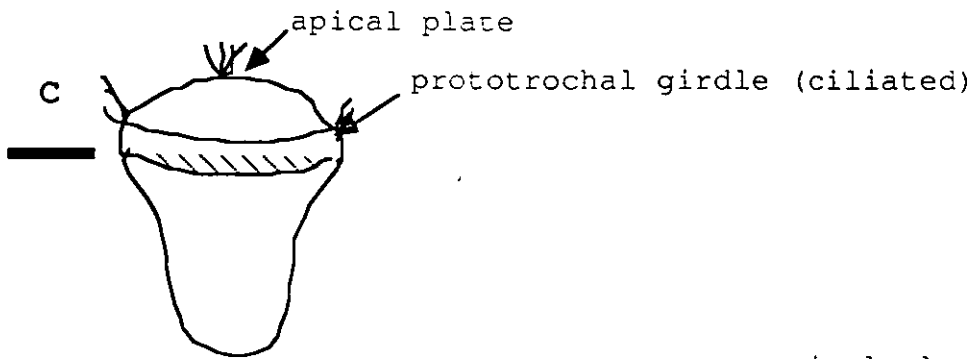
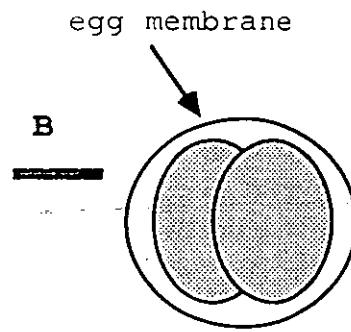
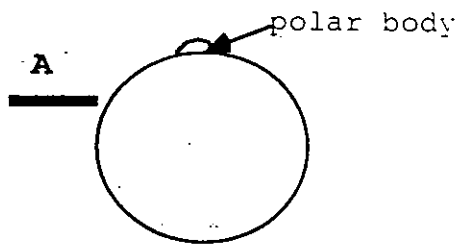
D. 'D-shaped' veliger larva, with valves open and velum extended. The velum is totally withdrawn when the valves are closed.

Bar = 30  $\mu\text{m}$ .

E. 'Umbone' veliger larva with valves open and velum extended. This stage occurs between approximately 120  $\mu\text{m}$  and 300  $\mu\text{m}$  (ready-to-set stage).

F. Ready-to-set pediveliger larva with velum extended and foot protruding. The velum and foot are totally withdrawn when the valves are closed.

Bar = 100  $\mu\text{m}$ .



the time when they are ready to settle is largely controlled by patterns of water movement within the water column (Meadows and Campbell 1972). However, the final choice of a place to settle is not usually left to chance.

A wide range of environmental factors has been found to stimulate settlement and metamorphosis of marine invertebrate larvae, with larvae of different species responding to a variety of non-biological (e.g. surface texture, roughness, contour, colour, background illumination) and biological (chemicals released by other animals, plants and bacteria) stimulants (Crisp 1965, 1967, 1974, Meadows and Campbell 1972, Scheltema 1974). If competent larvae do not encounter the correct stimulus as soon as they are ready for settlement, they will often enter a prolonged searching stage. Thus settlement and metamorphosis may be delayed (for one or two weeks if necessary) until a specific signal is received (Crisp 1965, Morse and Morse 1984b, McGrath *et al.* 1988).

The role of bacterial films on submerged surfaces in the induction of settlement and metamorphosis of various species of marine invertebrate larvae has been studied. Although biological films seem to inhibit the settlement of some species (Crisp and Ryland 1960), it is clear that the majority of larvae studied to date settle more readily on filmed surfaces.

'Unfortunately, there have been very few studies of the influence of bacteria on the settlement and metamorphosis of marine molluscan larvae (Table 6). It is probable that bacterial biofilms play important roles in the settlement and metamorphosis of other molluscan species. Numerous papers report the presence of substratum-associated chemicals which have been shown to induce settlement (Scheltema 1961, Crisp 1967, Veitch and Hidu 1971, Hadfield 1977, Morse *et al.* 1980, Seki and Kan-no 1981, Rumrill and Cameron 1983, Morse and Morse 1984a,b, Morse *et al.* 1984, Petersen 1984). The chemical components responsible were not identified

but it is possible some were of bacterial origin. It is therefore likely that bacteria do play a role in the induction of settlement of some of the molluscs studied by these authors. Settlement of abalone larvae has been shown to be induced by chemicals present at the surface of crustose coralline algae (Morse and Morse 1984b). The bacterial biota of coralline algae has been found to be very stable, and to be dominated by bacteria of the genus *Moraxella* (Lewis *et al.* 1985). However there is no published data that these bacteria induce settlement of competent abalone larvae on coralline algae.

## 2.0 Provision of Nutrients.

Marine bacteria are known to release organic compounds into the surrounding water. Although the rate and type of compounds released is dependent on the species of bacteria, availability of nutrients to bacteria and culture conditions, the main products appear to be carbohydrates and lipids (Goutx *et al.* 1987). The amount of dissolved organic matter excreted by marine bacteria is low (about 200  $\mu\text{g}$  per mg bacterial C day<sup>-1</sup>) and therefore usually has no significant influence on the daily energy budget of oceanic ecosystems. However inputs of dissolved organic compounds through bacterial activity may have significant ecological effects in areas where bacterial biomass is concentrated (e.g. at air/water or solid/water interfaces) (Goutx *et al.* 1990).

Embryos and planktonic larvae of some marine molluscs have been shown to be able to absorb organic nutrients directly from the water column (Crane *et al.* 1957, Frankboner and deBurgh 1978, Rice *et al.* 1980, Manahan and Crisp 1982, Manahan 1983a,b, Manahan *et al.* 1983, Manahan *et al.* 1989). Kinetic evidence that the rate of uptake of these compounds from natural seawater is significant to the energy budget of larvae has been documented (Manahan 1983a, Manahan *et al.* 1989). Most



Table 6. Marine mollusc larvae which are stimulated to settle and metamorphose by bacterial biofilms.

Mollusc species	Source of stimulation	Reference
<i>Ostrea edulis</i>	natural biofilms	Cole and Knight-Jones 1949 cited in Scheltema 1974
<i>Nassarius obsoletus</i>	sediment bacteria	Scheltema 1961
<i>Janua (Dexiospira) brasiliensis</i>	natural biofilms	Kirchman <i>et al.</i> 1982 a,b
<i>Haliotis rufescens</i>	cyanobacteria cell-free extract	Morse <i>et al.</i> 1984
<i>Crassostrea gigas</i>	mono-culture biofilm	Weiner <i>et al.</i> 1985

of these studies have been carried out in the laboratory using simple molecules (usually amino acids or glucose) in solution.

Very few studies reporting uptake of complex, naturally occurring dissolved organic compounds have been published. Amouroux (1984) compared the uptake of dissolved compounds excreted by microalgae and bacteria, by adults of the bivalve *Venus verrucosa*. It was found that 62% of bacterial exudate and 20% of microalgal exudate were adsorbed by the animals. The rate of uptake was sufficient to satisfy a significant portion of the total energy budget of the bivalve. In this study, bacterial and algal exudates were collected and "preserved" by sterilisation at 110°C for 10 minutes. It is likely that heat-labile compounds present in the exudates would have been denatured by this treatment. Thus the range of compounds tested for uptake by the bivalves would have differed from those initially secreted by the bacteria and microalgae. Nonetheless, it is reasonable to assume that larval stages of this and other mollusc species are able to absorb dissolved substances of bacterial origin. The ecological significance of such uptake needs to be investigated.

There is evidence that bacterial cells can be utilised to some extent as food by adult, filter feeding marine molluscs (Zobell and Feltham 1938, Harvey and Luoma 1984). Additionally, high natural agglutinin levels are present in the mantle, gills and digestive diverticula of adult oysters *Crassostrea gigas* (Mori *et al.* 1980), indicating the ability of these animals to agglutinate certain bacteria in the gut after ingestion. The possibility that larvae of these molluscs are also able to derive some nutritional benefit by lysing agglutinated bacteria was not investigated.

## **E. Causes of Mortalities of Marine Bivalve Larvae.**

In unpolluted conditions in estuaries and near-shore areas, the factors which limit survival and growth of marine bivalve larvae include the following (Mackenzie 1981, Tettelbach and Rhodes 1981, Valiela 1984):

- i) abundance of suitable food;
- ii) prevalence of predators;
- iii) marked fluctuations in environmental parameters such as temperature, salinity, dissolved oxygen, turbidity and water movement (lateral and upwelling).

Tubiash and Otto (1986) suggest that when conditions in the wild favour both spawning of oysters and bacterial proliferation in the water column (e.g. during the summer months), natural epizootics of bacterial disease may reduce larval survival rates. However, no studies of epizootics of bivalve larvae in natural environments have been published, and the significance of larval losses due to bacterial disease in these environments has not been clearly established.

In the hatchery, the causes of mortalities of marine bivalve larvae appear to be quite different for two major reasons. Firstly, larval losses due to sub-optimal culture conditions are much lower. The culture of bivalve larvae in high density conditions, as practiced in many hatcheries throughout the world (Holliday 1985), involves optimising environmental conditions (e.g. food types and levels, water quality, water temperature). Most bivalve larvae prefer live microalgae as a food source. Therefore mass cultured microalgal cells are usually added to the larval cultures, since intake-seawater usually contains inadequate levels of these organisms

(Guillard 1983, Brown *et al.* 1989, Jeffrey *et al.* 1990). Seawater-based media for larval culture is generally filtered to at least 10  $\mu\text{m}$  to reduce predators. The temperature of the culture medium often needs to be raised because the temperature of intake-seawater <sup>in temperate regions</sup> is usually sub-optimal.

The second reason relates to the increased abundance of bacteria in hatcheries. The optimal conditions for rearing animals also favour the proliferation of bacteria (Murchelano *et al.* 1975). For example, bacterial multiplication in water used for the culture of bivalve larvae is favoured by the elevated temperature at which larvae are usually reared and also by the increased nutrient levels present in the microalgal cultures fed to the larvae. Additionally, bacteria resident in shellfish hatcheries are largely protected from the ultra-violet component of direct sunlight, a factor which is known to limit their numbers in natural habitats (Sieracki and Sieburth 1986).

Apart from hygiene practices (discussed later) few factors limit bacterial abundance in hatcheries. Certain species of bacteriovores may be present in the hatchery system, if they are able to pass through 10  $\mu\text{m}$  water filters. Fenchel (1982) found that bacteriovores in the size range 2-10  $\mu\text{m}$  have a significant impact on bacterioplankton populations in the wild. Note that Fuhrman and McManus (1984) provided preliminary evidence that some bacteriovores in coastal waters can pass through 0.6  $\mu\text{m}$  filters.

## 1.0 Bacterial Diseases of Hatchery-Reared Marine Bivalve Larvae

As explained above, the relative importance of bacterial disease is much greater in hatcheries than in the wild as larval losses due to sub-optimal culture conditions are minimised and bacterial abundance in the larval culture media is greatly increased. Indeed, bacterial disease is often the major factor limiting survival of larval molluscs reared in intensive

culture systems. Bacteria of the genera *Vibrio*, *Pseudomonas*, and *Alteromonas* have been identified as the principle agents responsible for many of the mortalities (Table 7). A study of larval mortalities caused by mixed populations (Garland *et al.* 1986) contrasts to other reports, in which only single strains of bacteria were tested for pathogenicity. It is possible that two or more strains of bacteria, while not pathogenic in pure culture conditions, may act synergistically in mixed cultures to cause larval mortalities.

The types of bacterial pathogens of marine bivalve larvae are not clearly defined in the literature. For the purposes of this review the following definitions, based on Elston (1984) and Garland (1988) will be used.

- i) **Obligate pathogens:** very host-specific, multiply only in host cells, affect healthy hosts.
- ii) **Primary pathogens:** moderately host-specific, multiply to some extent in natural environments, affect healthy hosts.
- iii) **Opportunistic pathogens:** not particularly host-specific, multiply readily in natural environments, tend to affect only stressed or compromised hosts.

Most bacteria pathogenic to marine bivalve larvae are opportunistic pathogens, and require conditions favourable to their own proliferation in the hatchery system to cause disease (Elston 1984). These bacteria occur naturally in the marine environment (e.g. Murchelano and Brown 1969, 1970, Tubiash *et al.* 1970, Murchelano *et al.* 1975, Brown 1981b) but

Table 7. Bacteria associated with mortalities of experimentally-reared marine bivalve larvae.

Bivalve species <sup>a</sup>	Aetiological agent	Reference
<i>O. edulis</i>		Walne 1958
<i>M. mercenaria</i>	<i>Vibrio</i> sp. <i>Pseudomonas</i> sp.	Guillard 1959
<i>C. virginica</i> <i>O. edulis</i> <i>M. mercenaria</i>	<i>V. alginolyticus</i> <i>V. anguillarum</i> <sup>b</sup>	Tubiash <i>et al.</i> 1965, 1970
<i>C. virginica</i>	<i>Vibrio</i> sp. <i>Pseudomonas</i> spp.	Brown 1973
<i>M. mercenaria</i>	<i>Pseudomonas</i> spp.	Brown 1974
<i>C. virginica</i>	<i>Vibrio</i> sp. <i>Vibrio</i> sp. <i>Vibrio</i> sp.	Brown and Losee 1978
<i>O. edulis</i>	<i>V. anguillarum</i> <sup>b</sup>	DiSalvo <i>et al.</i> 1978
<i>C. virginica</i> <i>O. edulis</i>	<i>Vibrio</i> sp.	Blogoslawski <i>et al.</i> 1980
<i>C. virginica</i>	<i>Vibrio</i> sp.	Elston and Liebovitz 1980
<i>C. virginica</i>	<i>V. anguillarum</i> <sup>b</sup> <i>Pseudomonas</i> spp. <sup>4</sup>	Brown 1981a, b
<i>C. virginica</i>	<i>Vibrio</i> spp.	Elston <i>et al.</i> 1981

Table 7 continued.

<i>C. virginica</i>	<i>V. alginolyticus</i>	Elston <i>et al.</i> 1982
<i>O. edulis</i>		
<i>M. mercenaria</i>		
<i>C. gigas</i>	<i>Vibrio</i> spp.	Jeffries 1982
<i>C. virginica</i>	<i>Vibrio</i> spp.	Brown 1983
	<i>Pseudomonas</i> spp.	
<i>C. gigas</i>	<i>Vibrio</i> spp.	Garland <i>et al.</i> 1983
<i>C. gigas</i>	mixed population	<b>Garland <i>et al.</i> 1986</b>
<i>O. edulis</i>	<i>Vibrio</i> spp.	Nottage and
<i>C. gigas</i>		Birkbeck 1986
<i>C. virginica</i>	<i>Vibrio</i> sp.	Brown and
<i>M. mercenaria</i>		Petti-Tettlebach 1988

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<sup>a</sup>: Key to bivalve species:

*O. edulis* - *Ostrea edulis* , European flat oyster.

*M. mercenaria* - *Mercenaria mercenaria* , hard clam.

*C. virginica* - *Crassostrea virginica* , American edible oyster

*C. gigas* - *Crassostrea gigas* , Pacific oyster

<sup>b</sup>: also known as *Listonella anguillarum* (MacDonell and Colwell 1985) but see Nearhos and Fuerst (1987).

would not, under natural conditions, be expected to represent a threat to larvae.



## 1.1 Mechanisms of Infection.

Investigations of the mechanisms by which bacteria are pathogenic to bivalve larvae have revealed that disease is caused by either direct invasion of the host tissue or by production of extracellular toxins or by a combination of the two factors.

### 1.1.1 Direct Invasion.

Early reports of direct invasion of larval tissues by pathogenic bacteria were limited to largely circumstantial evidence and contained little histological study of healthy and moribund larvae. Brown (1973) found that a proportion of bacteria isolated from marine environmental samples ~~adversely affected different developmental stages of *C. virginica* and *M. mercenaria* larvae.~~ As neither filtrate from broth cultures nor heat-killed bacterial cells affected development of the larvae it was concluded that, in this case, invasion of the larvae by bacteria was necessary to cause disease. Similar conclusions were drawn by Guillard (1959) and Tubiash *et al.* (1965). However, there appears to have been a large variation in the quantity of extracellular bacterial metabolites to which the larvae were exposed in these three studies; discrepancies in the interpretation of results by Guillard (1959) were also apparent. Guillard (1959) exposed larvae to a cell-free broth equivalent to  $10^9$  cells mL<sup>-1</sup>. Surprisingly Guillard (1959) placed no importance on the emaciation and loss of feeding response by a group of larvae exposed to this cell-free broth, discounting the effect as being "due to the high concentration of metabolites". Instead



the author noted no negative effect on larvae exposed to either a whole-cell suspension of  $10^6$ - $10^7$  bacteria  $\text{mL}^{-1}$  or a cell-free filtrate from a duplicate broth culture, both of which had been treated with a combination of antibiotics.

--Tubiash *et al.* (1965) challenged larvae of *O. edulis*, *M. mercenaria*, *Aequipecten irradians* and *Teredo navalis* with whole-cell suspensions of washed bacteria isolated from moribund larvae and cultured on solid media. He concluded that invasion was necessary for the induction of disease for two reasons. Firstly, histological examination revealed bacteria present in tissues of moribund larvae. The second reason was that abnormal larvae, which were not able to feed, were the last (over time) to show signs of disease. Tubiash *et al.* (1965) did not challenge larvae with cell-free extracts from bacterial broth culture and so the possibility of disease being caused by bacterial exotoxins was not discussed.

Elston (1980) published a detailed guide to the ultrastructure of the oyster *C. virginica*, which facilitated histological examinations of the process of bacterial disease. Elston and Liebovitz (1980) and Elston *et al.* (1982) reported a form of pathogenesis caused by the direct invasion of host (*C. virginica*, *O. edulis* and *M. mercenaria* larvae) tissues by bacteria. The pathogens described by Elston *et al.* (1982) showed a strong affinity for surfaces. Evidence suggested that the conchiolin-containing ligament and periostracum of the larvae became infected after the larvae came into contact with a surface colonised by the pathogens. Invasion of larvae reported by Elston and Liebovitz (1980) occurred while the larvae were swimming in the water column, although infected larvae quickly became sedentary, resulting in the larvae becoming exposed to the high bacterial levels typically associated with the bottom of the larval rearing vessels.

The invasion and subsequent colonisation of the inner shell and adjacent mantle tissue by the bacteria resulted in the progressive disruption of mantle tissue and disturbance of associated regulatory activities. The bacteria also appeared to prevent normal shell deposition and ligament growth. It was also suggested by Elston and Liebovitz (1980) and Elston *et al.* (1982) that acidic bacterial metabolites may interfere with the normal deposition of calcium carbonate in newly elaborated shell, although this was not further investigated. Such disruptions rapidly led to the death of the larvae.

#### 1.1.2 Toxic Metabolites.

Mortalities of bivalve larvae caused by extracellular bacterial metabolites have been described more often than those caused by direct invasion of tissues. Brown (1981b) reported that the degree of pathogenicity of bacteria to larvae is dose dependent, (even providing evidence that extremely low doses of a known pathogen aided the development of larvae).

Different modes of pathogenesis mediated by external bacterial metabolites have been described in *C. virginica* larvae exposed to two *Vibrio* spp. (Elston and Liebovitz 1980). The first involves the formation of velar abnormalities in young larvae which resulted in decreased motility, decreased feeding activity and compromised levels of normal respiratory gas and metabolite exchange. The larvae remained active and this resulted in rapid depletion of food reserves and death. It was suggested that cleavage, by bacterial enzymes, of the proteins which mediate cell adhesion resulted in the detachment of velar cells. Elston and Liebovitz (1980) proposed that if external bacterial metabolites were responsible for the disease, they acted on the surface of the velar cells.

A second type of pathogenesis has been attributed to external bacterial metabolites by Elston and Liebovitz (1980). Initially larvae become inactive in the presence of suspended *Vibrio* sp. cells. This results in diminutions of food intake, respiratory gas exchange and regulatory activities. Inactive larvae are also exposed to the increased bacterial levels which occur at the bottom of the larval culture tanks. Results indicate that bacterial antigens attach to the cell coat of the larval digestive tract and are associated with the detachment of absorptive cells. Thus assimilation of nutrients is inhibited and various internal organs atrophy rapidly.

In both types of pathogenesis, bacterial invasion was observed late in the disease process. A similar pattern of infection was described by Brown (1983), viz invasion is a secondary phase of infection with bacteria becoming invasive only after larvae become morbid.

Tubiash *et al.* (1965) and Brown (1981b) both reported a granular appearance of internal organs of sick larvae under the dissecting microscope. Elston and Liebovitz (1980) suggested this granularity was due to a change in the transparency of internal organs caused by the detachment and breakdown of host cellular material.

There have been few detailed attempts to characterise extracellular bacterial metabolites which are pathogenic to marine bivalve larvae. In a semi-quantitative study, Brown (1983) described the toxicity of a *Vibrio* exotoxin. The addition of 6  $\mu\text{L}$  of toxin (produced by approx.  $10^4$  cells) to 1 L culture water did not inhibit the transformation of fertilised eggs into D-shape larvae. Mortality occurred after the larval stage of development was reached. In contrast the addition of 19  $\mu\text{L}$  toxin (produced by approx.  $10^6$  bacterial cells) (*sic*) inhibited normal development of fertilised eggs. These results indicate that a range of concentrations of bacterial extracellular metabolites should be tested before making any conclusions regarding their toxicity to bivalve larvae.

Brown and Petti-Tettelbach (1988) reported a non-motile *Vibrio* sp. which produced a heat-stable metabolite capable of inducing disease in clam and oyster larvae. Cell-free filtrates of broth cultures of this bacterium were able to cause disease in larval clams before or after being heated to 65°C for 30 minutes. Live washed cells of this bacterium could produce disease in the larvae at concentrations as low as 10 cells mL<sup>-1</sup> culture water. However, a similar level of heat-killed cells of the bacterium had a significantly lower effect on larval development. This indicated that the toxin was produced by actively metabolising cells. Another heat-stable compound, toxic to *O. edulis* and *C. gigas*, was found to be produced by a number of *Vibrio* spp. (Nottage and Birkbeck 1986).

Several authors (Brown and Losee 1978, DiSalvo *et al.* 1978, Jeffries 1982, Nottage and Birkbeck 1986) found the toxic metabolites of *Vibrio* spp. isolated from sick larvae and post-larvae were heat-labile, suggesting that the toxins involved may be proteins. Several studies (Brown and Roland 1984, Nottage and Birkbeck 1987a,b) have since shown that some toxic extracellular metabolites of *Vibrio* strains are proteinases.

Brown (1981a) reported that a red pigment, produced by a marine *Pseudomonas* sp., isolated from moribund *C. virginica* larvae, induced disease in normal larvae. The pigment was identified as being very similar to prodigiosin, an antibacterial, antifungal and antiprotozoan compound (Gerber 1975) which has been isolated from other marine bacteria (Lewis and Corpe 1964). Brown (1981a) found that a minimum dose of 3.4 µg pigment mL<sup>-1</sup> of culture water (produced by approx. 4.8 x 10<sup>6</sup> bacterial cells) was needed to elicit a toxic effect. This was in contrast to earlier findings by the same author (Brown 1974) which suggested the pigment itself was not toxic to *M. mercenaria* larvae, but that a level of 10<sup>3</sup> cells of the red-pigmented pseudomonad mL<sup>-1</sup> larval culture medium caused significant increases in abnormal larval development.

Very little work has been done to attempt to link specific biochemical traits of bacteria with pathogenicity of bivalve larvae. Jeffries (1982) reported the ability to ferment sucrose and the ability to degrade xanthins individually correlated with pathogenicity of three strains of *Vibrio* towards *C. gigas* and *O. edulis* larvae. Clearly, further studies are needed to establish whether bacteria pathogenic to marine bivalve larvae have any specific or common biochemical characteristics.

### 1.2 Larval Resistance to Bacterial Pathogens.

Brown (1973) suggested that larval oysters (*C. virginica*) become less susceptible to bacterial disease as they develop from embryos to veligers to ready-to-set larvae. The author attributed the increased resistance to either increased protection by the shell or an increased tolerance to the pathogens. The first reason may apply only in limited circumstances because, under normal conditions, larvae need to have their shell open in order to swim and feed. It is possible that larval tolerance to bacterial pathogens increases with age. Larger larvae have a greater store of food reserves with which to survive periods of declined feeding activity, such as those induced by some types of pathogenesis. Liebovitz and Elston (1980) found that oyster larvae which had experienced a limited mortality rate within 24 hours following a single exposure to a pathogenic *Vibrio* sp. suffered no significant further mortality after the 24 hour period. This indicates the ability of larvae to recover from bacterial infection.

Garland *et al.* (1986) found that when levels of mixed bacterial populations in larval culture medium reached approximately  $10^6 \text{ mL}^{-1}$  survival of larval *C. gigas* decreased significantly. This suggests that

there may be a threshold level of bacteria above which larvae are not able to survive.

### 1.3 Sources and Control of Bacteria Pathogenic to Marine Bivalve Larvae in Hatcheries.

Bacteria pathogenic to larval marine bivalves can enter a hatchery from any of 3 primary sources: intake seawater (Brown 1981b), broodstock (Jeffries 1982) and cultures of microalgae used as food for the developing larvae (Guillard 1959, 1983, Murchelano *et al.* 1975). Pathogens can also enter hatchery seawater at many points in the system, via contaminated equipment or materials (Elston 1984). Other obvious sources of pathogenic bacteria are sea-spray and the hands and clothing of hatchery staff; studies of the influence of these sources are very limited.

As mentioned above, all cases of bacterial diseases of marine bivalve larvae reported to date are considered to be caused by <sup>opportunistic</sup> pathogens, which would be found living freely, albeit at very low levels, in the hatchery system (Tubiash *et al.* 1965, Brown 1981b, Elston 1984, Brown and Petti-Tettelbach 1988). For these bacteria to be maintained at low levels, at which they represent no threat to larvae, practical cleaning regimes must be implemented in hatcheries. Elston *et al.* (1981) and Elston (1984) suggested routine bacteriological sampling to gain an understanding of the dynamics of bacterial populations in the system. The authors also outlined simple procedures for disinfection of culture containers and water supply lines. Such procedures are easily incorporated into the daily operations of a commercial hatchery as described in Lewis *et al.* (1986).

Methods which have been used to reduce populations of bacteria in seawater and on surfaces in marine shellfish hatcheries include filtration (usually to 10  $\mu\text{m}$ ) (Holliday 1985) followed by exposure to ozone gas or silver nitrate (Blogoslawski *et al.* 1978), ultra-violet light irradiation

(Brown and Russo 1979), chlorination or antibiotic treatment (D'Agostino 1975) and heating to  $> 60^{\circ}\text{C}$  (Murchelano *et al.* 1975, Lewis *et al.* 1988).

Unfortunately, these procedures can often produce undesired side-effects. Antibiotics, when administered in low doses, have been shown to improve embryonic development of oysters (Hidu and Tubiash 1963, Murchelano *et al.* 1975, Brown 1983). However, inappropriate administration of antibiotic compounds may induce the proliferation of strains of antibiotic-resistant bacteria, or the compounds may be directly toxic to larvae (Walne 1958, D'Agostino 1975, Provasoli 1977, Tubiash and Otto 1986, Brown and Petti-Tettelbach 1988); hypochlorite, silver nitrate and ozone can inhibit normal larval development (Blogoslawski *et al.* 1978, Richardson *et al.* 1982); ultra-violet light irradiation does not necessarily kill all bacteria in seawater (Murchelano *et al.* 1975, Tubiash 1975, Brown 1981b, Spotte and Adams 1981).

Murchelano *et al.* (1975) suggested that control of the total bacterial load in oyster larval culture water is an important factor in reducing bacterial diseases of oyster larvae. There is a clear need for methods which are aimed at reducing sources of bacterial contamination in hatchery systems, rather than inhibiting the proliferation of bacteria once they have entered the culture medium. The identification of sources of bacteria pathogenic to shellfish larvae and the control of the total bacterial populations in those areas is critical to successful production of bivalve larvae. This approach formed the basis of the studies described in the following sections of this thesis.

## MATERIALS AND METHODS

### Introduction.

The procedures outlined in this section have been divided into 2 main areas. A) The design and daily running of the commercial Pacific oyster (*Crassostrea gigas*) hatchery in which the study was undertaken, at Bicheno, Tasmania, Australia (41°53' S, 148°16' E).

B) The materials and methods used during bacteriological monitoring at the hatchery.

As mentioned in the general introduction the major aim of this study was to identify and rectify the causes of massive mortality of oyster larvae within the hatchery. Consequently, during the course of this study, numerous small alterations were made to the operational systems of the hatchery as results of bacteriological tests indicated the need for change.

Changes were suggested on the basis of scientific results obtained, but often the protocols adopted also took into account the management viewpoint. Therefore the most beneficial changes, from a bacteriological perspective, could not always be accomplished due to considerations such as cost, disruption to production schedules and operational **practicability**. Those changes that were made are detailed, in order, under the description of each system within the hatchery.

### A. Hatchery Operations.

As the procedures used in the operation of the hatchery are pertinent to the nature of this study, they are described in detail in this section.

The general operations in the hatchery were separated into 3 areas. These were the provision of adequate supplies of fresh, clean seawater to the hatchery; the culture of specific clones of microalgae as food for



oyster larvae and broodstock and the production and rearing of oyster larvae.

### 1.0. Seawater Delivery System.

Due to the intensity of the culture system employed at the hatchery, seawater used in the hatchery needed to be of very high quality. This was achieved by a combination of biological and physical filtration as described below.

#### 1.1 Initial Treatment of Seawater.

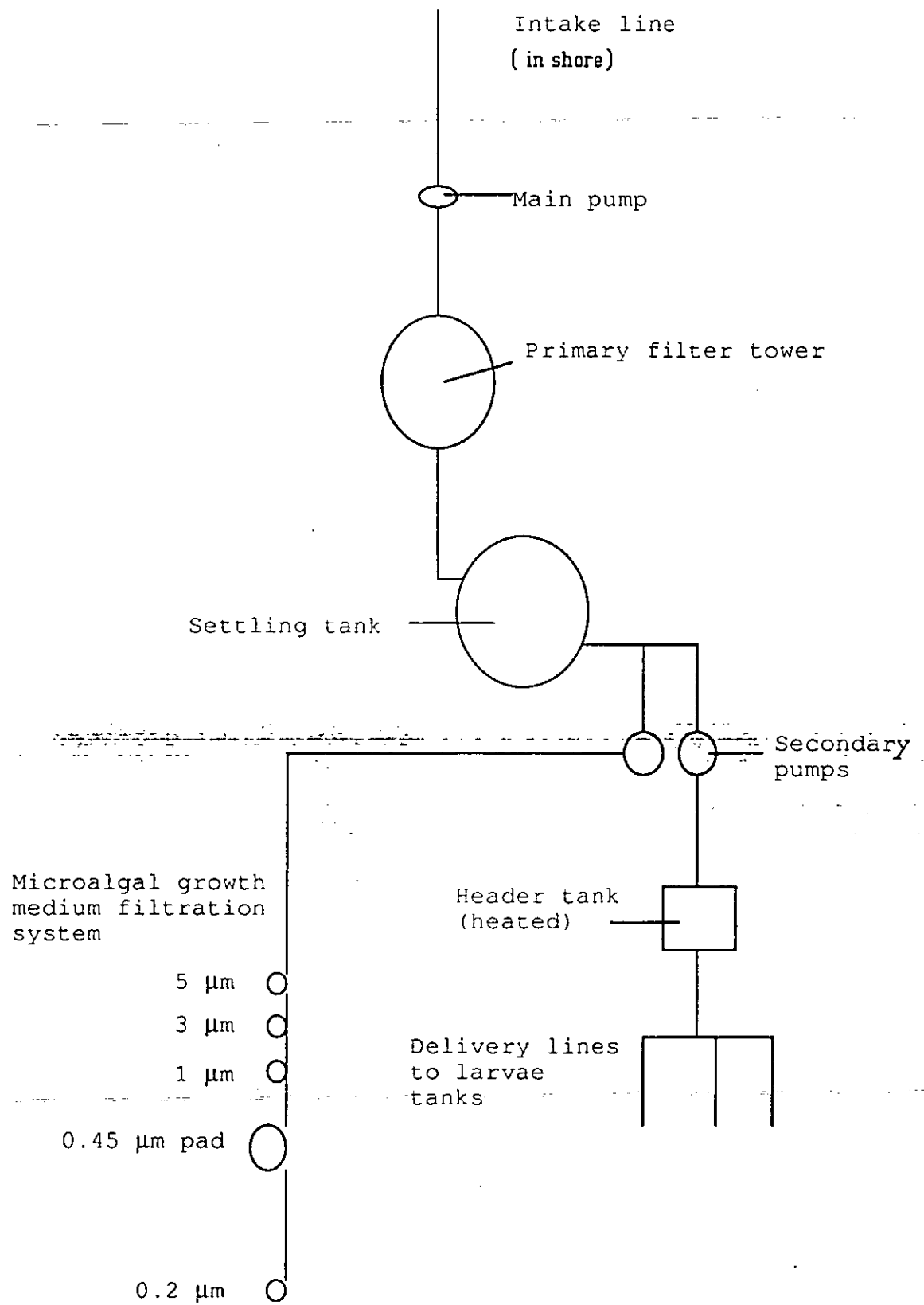
Fresh seawater for use within the hatchery was pumped from above a sandy bottom in an area sheltered by a near-shore island. The water was pumped through approximately 100 m of poly-ethylene and poly-vinyl chloride (P.V.C.) pipe into the primary filtration tower (Figure 2). This tower had an internal height of 3.5 m and a diameter of 1.8 m.

Seawater was pumped into the top of the tower and from there flowed through a series of graded rock layers into a collector pipe at the bottom of the tower. The finest rock layer used in the tower consisted of a 10 cm-thick layer of 5-7 mm (largest diameter) stones, which removed particles between 300-500  $\mu\text{m}$  in diameter from the incoming water.

In addition to acting as a mechanical filter, the tower also provided a large surface area for microbial colonisation and activity. If nitrifying bacteria were present, as is normal (Spotte 1970), it can be assumed that efficient oxidation of toxic nitrogenous compounds occurred, as flow rates were approximately 2-6 filter-bed volumes  $\text{hour}^{-1}$  (i.e. 20-50  $\text{m}^3\text{h}^{-1}$ ) (Mayo 1976).

The tower was backflushed when necessary to remove accumulated particulate matter. After backflushing water was passed through the filter

Figure 2. Hatchery Seawater System.



and run to waste for approximately 30 min to clear the filter of any loose material.

After passing through the filter tower the seawater was directed into a settlement tank. This tank was circular with a diameter of 2 m and a depth of 1 m. The water was directed into the tank to create a circular flow. This served two purposes:

- a. It concentrated any particulate matter in the water into the centre, away from the outflow pipe.
- b. The continuous water flow stimulated growth of filamentous algae on the tank walls and bottom which served as a secondary biological filter. The algae were allowed to grow until filaments started to break away and block the outflow pipe. When this occurred the tank was quickly drained and excess algae and particulate matter removed. The tank was refilled as soon as possible to prevent death of the benthic algae and small filaments still attached to the floor and walls of the tank.

## 1.2 Hatchery Seawater.

Water passing out of the settlement tank was divided into 2 systems. One supplied the larval rearing rooms and the other supplied water to the algal culture rooms.

### 1.2.1 Larval Growth Medium.

Seawater was pumped, on demand, from the settlement tank to the first floor of the hatchery where it was passed through a 25  $\mu\text{m}$  sieve (Nitex) and into a fibreglass header-tank (2 m x 1.5 m x 0.75 m deep). The water was pre-heated (24-28°C) in this tank before being delivered to the larval tanks through 50 mm diameter P.V.C. pipes.

The plumbing between the header tank and larval tanks was air dried between uses. This was achieved by forcing air from the hatchery air blower unit through the pipes.

### 1.2.2 Microalgal Growth Medium.

One of the major areas of modification during the period of this study was the system used to prepare seawater for the semi-continuous culture of microalgae to be used as food for the oyster larvae. Modifications were generally made between production seasons and can thus be described on a year-to-year basis.

From the start of the 1984/85 production season all exposed ends of filter apparatus and media transfer lines (see Materials and Methods section A.2.0) were routinely sprayed with an aqueous methylated-spirits solution [95% (v/v) ethanol, 0.25% (v/v) methyl isobutyl ketone; C.S.R.] before connecting all lines to and after disconnecting them from any other fitting during normal operations. This provided a high degree of disinfection of fittings between uses and minimised the chances of cross-contamination of microalgal cultures with bacteria via common fittings. Spraying sterile fittings immediately after removal from an autoclave bag reduced the chances of contamination by airborne microorganisms.

Methylated-spirits was also used as a surface disinfectant in other areas of the hatchery when clean conditions were required.

#### 1.2.2.1 1983/84 Production Season.

During the 1983/84 production season seawater for use as growth medium for microalgae was pumped from the settlement tank through a 1  $\mu$ m cartridge filter (AMF-Cuno) and into a series of covered, but not airtight, 500 L fibreglass tanks. Immersion heaters were used to pasteurise the seawater (85-90°C, 1 hr). Following pasteurisation the seawater was

allowed to cool, while still in the pasteurisation tanks, for 8-10 hr before being pumped through a titanium heat-exchanger to further cool the water, and into the 500 L microalgal culture bags (Materials and Methods section A.3.2.1) via a length (approximately 40 m) of plastic hose (Nalgene, food grade). The seawater was then allowed to cool for a further 24 hr before sterile microalgal growth nutrients were added and the bag inoculated with the microalgal starter culture (Materials and Methods section A.3.2.3).

The delivery line between the pasteurisation tanks and the 500 l bags was allowed to air dry between each use and disinfected every 2 weeks with a commercial disinfectant (Safsol) prepared at a concentration of 100 ppm free chlorine. Disinfection was achieved by filling the line with this solution and allowing it to stand for 1 hr before draining and air drying. Immediately prior to the first use after disinfection the delivery line was thoroughly rinsed with hot (50-60°C) pasteurised seawater.

Bacteriological studies carried out by Dr. C.D. Garland (University of Tasmania, Hobart) during this production season showed that the pasteurising/cooling (PC) method for reducing the numbers of viable bacteria in the microalgal growth medium was unsatisfactory. Preliminary bacteriological evidence obtained prior to the commencement of the present study indicated that bacteria in feed microalgal cultures were the agents responsible for the high mortalities of larvae and thus it was decided to replace the PC system with a membrane filtration system designed to deliver the required quantities of 0.2 µm membrane-filtered seawater.

#### 1.2.2.2 1984/85 Production Season.

It was decided to replace the pasteurisation technique of disinfecting the seawater growth medium for feed microalgae for two reasons. Firstly it was not practical to sterilize the delivery line from the pasteurisation

tanks to the 500 L microalgal culture bags. Hence without such sterilization, post-pasteurised seawater would be very likely to become contaminated with bacteria shedding from the inner surface of the delivery pipes. This is a typical microbiological problem in plumbing systems (Characklis and Cooksey 1983). Secondly such bacterial contaminants would be very likely to increase by  $1-2 \log_{10}$  units  $\text{mL}^{-1}$ , as the post-pasteurised seawater cooled over 24 hours. This increase is typical of marine bacteria in seawater (Zobell 1946). It was also considered desirable to develop a technique for the preparation of microalgal growth medium which was not as labour-intensive as the PC method.

To reduce the bacterial load of the seawater growth medium for feed microalgae,  $0.2 \mu\text{m}$  membrane-filtration (MF) was used. This technique was known to reduce the bacterial count of large volumes of liquid (Brock 1983), but had not been previously used for the treatment of seawater. Consequently all microalgae fed to larvae in 1984/85 were grown in 500 L bags filled (and refilled after harvest) with  $0.2 \mu\text{m}$  membrane-filtered seawater. The filtration unit was located sufficiently close to the 500 L bags to enable the use of a short length of seawater delivery tube, which could be sterilized (autoclaved) easily on a daily basis.

The 500 L bag cultures were inoculated with starter cultures of axenic microalgae during all of this season.

#### i.) Design of the $0.2 \mu\text{m}$ Membrane-Filtration System.

The first  $0.2 \mu\text{m}$  membrane-filtration system designed and installed for the production of microalgal growth medium consisted of the following elements:

- a. A series of 9.75" cartridge (depth) filters of 5, 3 and  $1 \mu\text{m}$  pore size respectively in poly-propylene housings (AMF -Cuno, Millipore etc).

b. A 16-cell pad filter of nominal 0.45  $\mu\text{m}$  pore size (AMF-Cuno model PTP-1).

c. A 9.75" cartridge 0.2  $\mu\text{m}$  (membrane) filter with a poly-propylene housing (Millipore model CVGL 01T P1).

To operate the membrane-filtration system all pre-filters were securely fastened in their housings and the housing for the 0.2  $\mu\text{m}$  membrane-filter cartridge was assembled without the cartridge being installed. A small steam generator was attached to the outlet end of the 0.2  $\mu\text{m}$  housing and low-pressure steam was passed through the filtration system for about 10 minutes. A sterile (autoclaved) 0.2  $\mu\text{m}$  membrane-filter cartridge was installed in its housing, which was still hot (60-80°C) after being steamed.

The end of a sterile (autoclaved) MTLA (see Materials and Methods section A.2.1.1) nearest the stainless-steel T-piece was freed from its autoclave bag, sprayed with methylated-spirits and connected to the outlet hose tail of the 0.2  $\mu\text{m}$  filter housing. The nutrient dispenser was connected to the T-piece as described in Materials and Methods section A.3.2.1.

Once assembled about 50 L of seawater was pumped to waste through the filtration system before any filtered seawater was used for microalgal culture purposes.

The procedure for cleaning and disinfecting this filtration system after use each day was as follows.

- All pre-filter units (cartridges and the pad filter) were removed from their housings, rinsed with hot (approximately 60°C) fresh tap water and air dried at room temperature.

- All filter housings were hand cleaned in hot tap water and air dried at room temperature.

- The 0.2 µm cartridge and the MTLA unit were rinsed with hot tap water and autoclaved separately in open autoclave bags. Immediately after autoclaving the bags were sealed (airtight) and stored in a dry cupboard until required.

To determine the integrity of the 0.2 µm filter membrane, samples of 0.2 µm membrane-filtered seawater were examined daily for the presence of viable bacteria (see Materials and Methods section B.1.1.1.1). This necessitated an incubation period of 5-7 days after the sample was taken before the integrity of the cartridge could be confirmed. Consequently a series of 7-8 0.2 µm filter cartridges was used on a daily rotation basis, to enable the bacteriological quality of the seawater delivered by a given filter to be analysed before that cartridge was used again. If a cartridge was thought to be faulty it was discarded and replaced by a new, sterile (autoclaved) cartridge.

This system yielded seawater with satisfactorily low bacterial numbers for about 2 months. Two factors then prompted a review of the disinfection procedures for the 0.2 µm filter cartridge and housing. The first was the high failure rate of 0.2 µm cartridges as indicated by the bacteriological testing of the 0.2 µm filtrate yielding high bacterial counts. This high failure rate (i.e cartridges failing after only 5 or 6 cycles) was found to be due to breakdown of the filter membrane caused by repeated



autoclaving (Millipore personnel, pers. comm.). Further consultation with that company resulted in the hatchery being supplied with a higher grade cartridge (Millipore model CWFG O1T PE) to replace the initial filter type. The new filters were designed to withstand repeated autoclaving and were not expected to lose their integrity within 15-20 cycles (Millipore personnel, pers. comm.).

The second problem was caused by the repeated steaming of the 0.2  $\mu\text{m}$  poly-propylene filter housings. Steam had caused the poly-propylene components of this housing to discolour and crack.

The disinfection process for the 0.2  $\mu\text{m}$  filter housing was changed to one of manual cleaning in hot (approximately 60°C) tap water with air drying at room temperature. However, this system proved to be inadequate to provide seawater of consistently low bacterial numbers.

#### 1.2.2.3 1985/86 Production Season.

Two major changes were made to the 0.2  $\mu\text{m}$  membrane-filtration and filter cleaning systems before the start of this production season. The first was the purchase of an autoclavable (stainless-steel) housing for the 0.2  $\mu\text{m}$  filter cartridges. The housing was fitted with attachments which allowed aseptic *in situ* integrity testing (bubble point method, Brock 1983) of the filter membrane.

The second change was the purchase of a domestic fan-forced oven. This was used for drying filter cartridges and pads at 50-60°C instead of at room temperature.

Before use each day a clean 0.2  $\mu\text{m}$  membrane-filter cartridge was installed in the stainless-steel housing. An MTLA unit was attached to the housing in the normal fashion and the exposed ends of the silicon hose and the stainless-steel T-piece were loosely capped with aluminium foil

(Comalco). The complete unit was autoclaved and left in the closed autoclave until required.

To prepare this revised seawater filtration system for operation the oven dried pre-filter cartridges and pad were installed in their respective housings. The sterilised 0.2  $\mu\text{m}$  filter-cartridge/housing/MTLA unit was removed from the autoclave and attached to the prefiltration system. The integrity of the 0.2  $\mu\text{m}$  filter membrane could then be tested. If the membrane proved to be faulty the cartridge was removed, all internal surfaces of the stainless-steel filter housing sprayed with methylated-spirits and a new sterile (autoclaved) 0.2  $\mu\text{m}$  filter cartridge installed. The integrity of new cartridges was always tested before use.

When an intact cartridge was in place, the foil caps were removed from the free ends of the nutrient dispensing line (Materials and Methods section A.3.2.1) and the stainless-steel T-piece of MTLA. The exposed ends were then sprayed with methylated-spirits and the hose fitted over the stainless-steel to form an air-tight seal. The seawater delivery system was now ready for operation.

## 2.0 Media Transfer Lines.

During the course of planning and setting up the microalgal culture system, staff at the hatchery had developed several hose assemblies to facilitate the transfer of media (liquid and gas) into and out of microalgal cultures. To simplify later descriptions of culture procedures the designs of all media transfer lines (MTLs) are given in this section.

All MTLs consist of a combination of some or all of the following components:

a. Silicon rubber hose. Internal diameter (i.d.) 11 mm; Wall thickness (w.t.) 1.5 mm.

b. Thick plastic hose (Nalgene, food grade). i.d. 10 mm; w.t. 1.5 mm.

c. Thin plastic hose (Nalgene, food grade). i.d. 4 mm; w.t. 1 mm.

d. Autoclavable air filters (Millipore 'Millex 50', 0.2  $\mu$ m pore size).

e. The external cylinder of a 10 mL plastic syringe (Terumo). (The thick plastic hose formed an airtight seal when inserted into the cylinder. Thin plastic hose formed an airtight seal when pushed over the nipple of the cylinder.)

f. Autoclavable plastic ball valves (Nalgene). The hose tails on these valves form airtight seals with both thick Nalgene hose and the silicon hose.

g. A stainless-steel T-piece hose joiner. The cross arm forms an airtight seal when inserted into the silicon hose. The perpendicular arm forms an airtight seal when inserted into thin Nalgene hose.

h. Pasteur pipettes (BRAND, 9 cm glass). Form an airtight seal when inserted into thin Nalgene hose.

## 2.1 Design of Media Transfer Lines.

The designs of the various media transfer lines are detailed below. All connections between fittings were airtight.

### 2.1.1 Media Transfer Line A (MTLA, Figure 3A).

a 5 m length of silicon hose attached to one end of the cross arm of the stainless-steel T-piece. A short (approximately 10 cm) length of thick Nalgene hose was connected to the other end of the cross arm.

### 2.1.2 Media Transfer Line B (MTLB, Figure 3B).

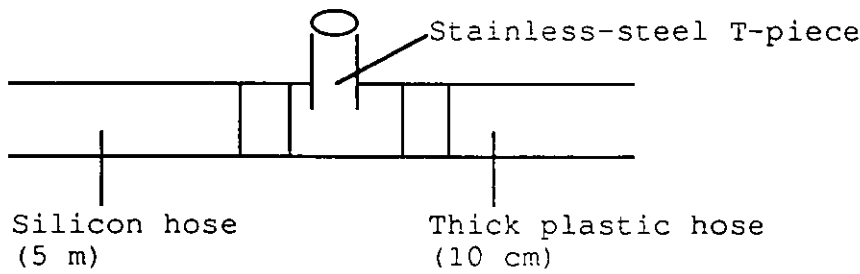
a syringe cylinder with the distal (nipple) end removed so as to form a cylinder of uniform bore. A short (approximately 10 cm) length of thick Nalgene hose was inserted into the proximal end of the cylinder and a 0.2  $\mu\text{m}$  air membrane-filtration unit fitted to the free end of the plastic hose.

### 2.1.3 Media Transfer Line C (MTLC, Figure 3C).

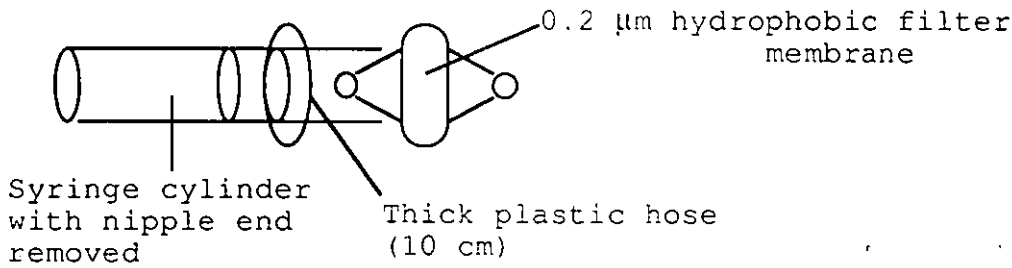
a length (approximately 2 m) of thin Nalgene hose attached to the nipple of a syringe cylinder. A short (approximately 10 cm) length of thick Nalgene hose was inserted into the open end of the syringe cylinder.

Figure 3. Media Transfer Lines for microalgal culture  
(not to scale)

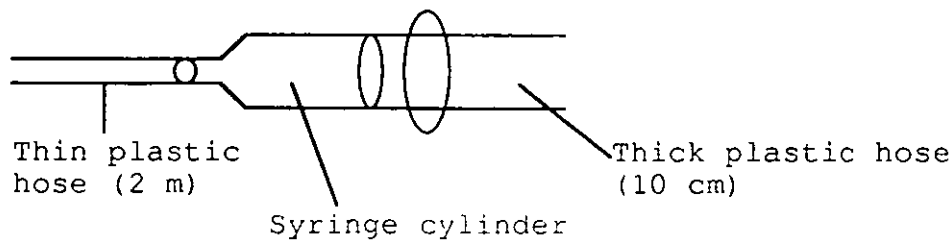
A. Media Transfer Line A



B. Media Transfer Line B



C. Media Transfer Line C



D. Media Transfer Line D

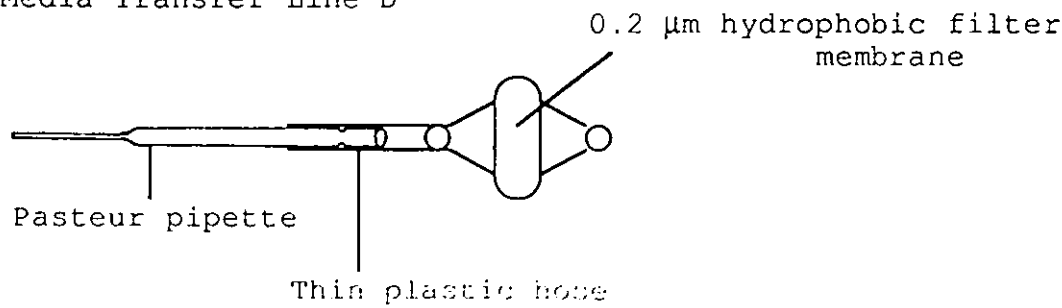
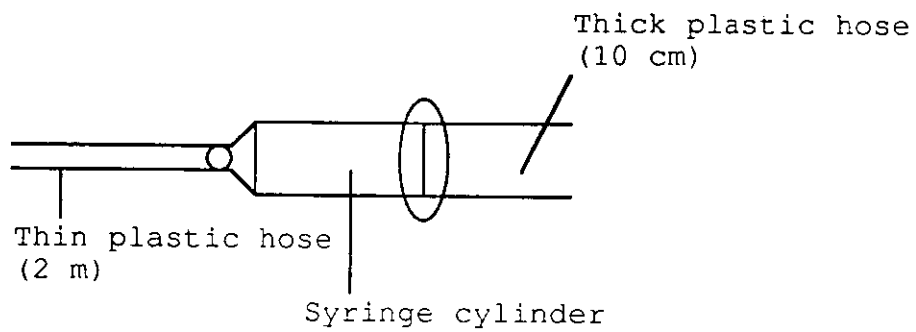
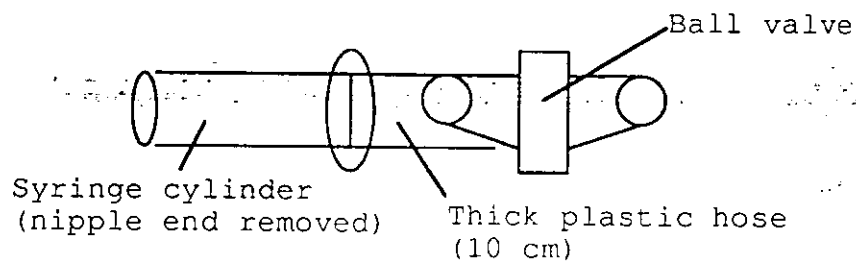


Figure 3 continued.

## E. Media Transfer Line E



## F. Media Transfer Line F



#### 2.1.4 Media Transfer Line D (MTLD, Figure 3D).

a pasteur pipette connected to a Millex 50 air filter by a short (approximately 5 cm) length of thin Nalgene hose.

#### 2.1.5 Media Transfer Line E (MTLE, Figure 3E).

a length (approximately 2 m) of thin Nalgene hose connected to a short (approximately 10 cm) length of thick Nalgene hose by a syringe cylinder.

#### 2.1.6 Media Transfer Line F (MTLF, Figure 3F).

a syringe cylinder with the nipple end removed as for MTLB. A short (approximately 10 cm) length of thick Nalgene hose was inserted into the proximal end of the cylinder. An autoclavable ball valve was attached to the free end of the Nalgene hose.

#### 2.1.7 Media Transfer Line G (MTLG).

a length (approximately 4 m) of thick Nalgene hose.

### 2.2 Preparation of Media Transfer Lines.

All MTL's were disassembled before use, rinsed with hot (approximately 60°C) tap water and reassembled. All open ends of hoses and fittings were capped with aluminium foil (Comalco) and the complete transfer lines placed individually into autoclave bags. Assembled transfer lines were autoclaved at 50 kPa for 30 min. Immediately after autoclaving the autoclave bags were sealed (airtight) and the sterile units stored in a dry cupboard until required.

### 3.0 Microalgal Culture Methods.

The successful production of large volumes of microalgae was, and still is, an integral part of operations at the hatchery. Small axenic monocultures of appropriate species were maintained on site. When required these cultures were sequentially subcultured into larger vessels in order to produce sufficient food for the oyster larvae.

Pure cultures of axenic (bacteria free) were obtained in 20 mL vials from R.R.L. Guillard, Maine, U.S.A. Strains used at the hatchery during the course of this study were as follows:

*Isochrysis* sp. (clone T. Iso)

*Chroomonas salina* (Butcher 3C)

*Thalassiosira pseudonana* (Hasle et Heimdal 3H)

*Chaetoceros calcitrans* Paulsen

*Dunaliella tertiolecta* Butcher

*Tetraselmis suecica* Butcher

*Pavlova (Monochrysis) lutheri* Droop

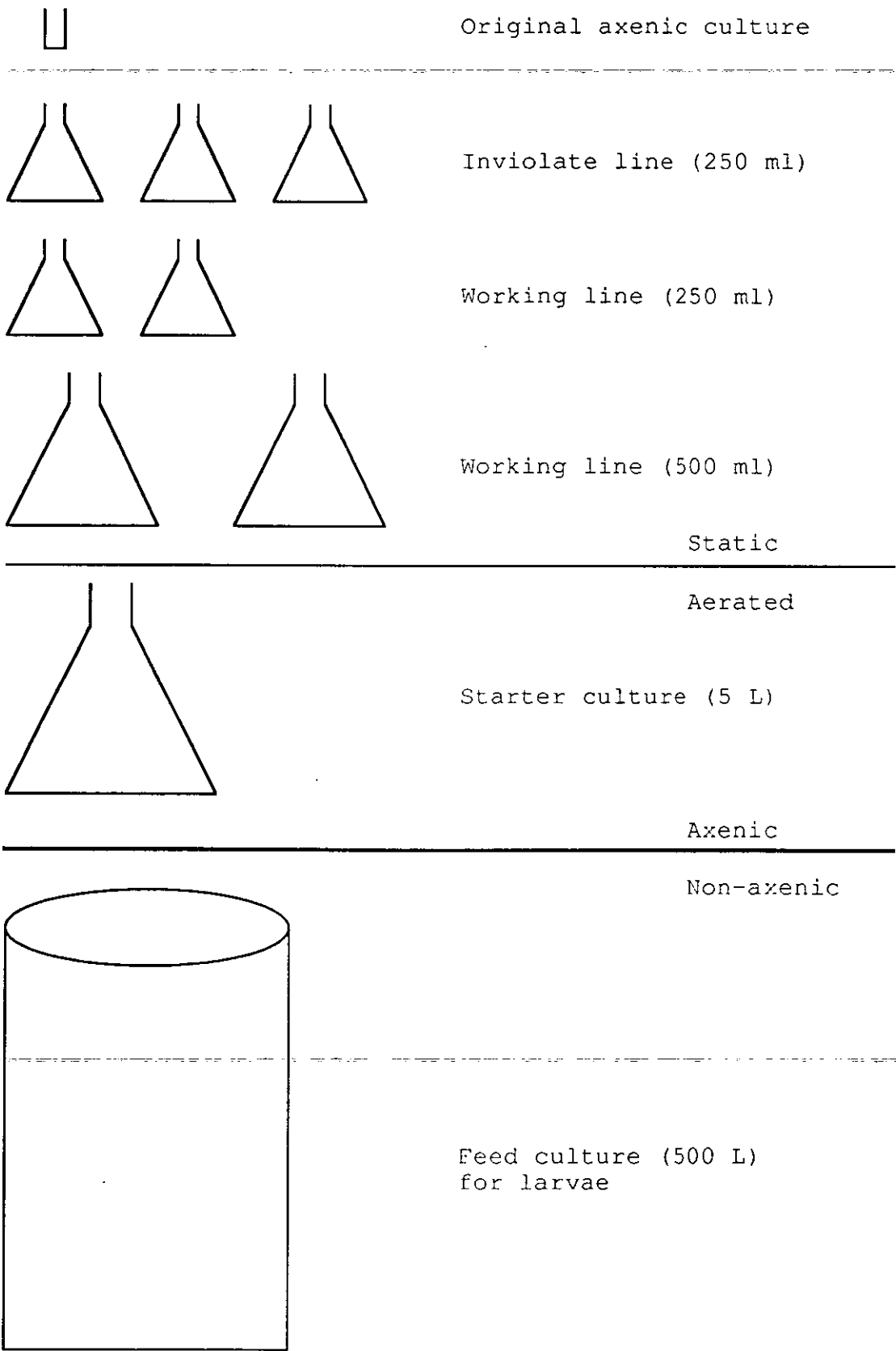
Immediately upon receipt, the contents of each 20 mL vial of culture were used to inoculate 2 x 250 mL flasks containing sterile growth medium as described in Materials and Methods section A.3.1.1.1, below.

### 3.1 Culture of Microalgae to the 5 L Flask Stage.

The subculture system used for propagation of microalgal cultures at the hatchery is shown in Figure 4. The system was designed to maintain all flask cultures up to and including the 5 L stage in an axenic (bacteria free) state. This was relatively easy to achieve as all culture flasks could be completely assembled and filled with growth medium before autoclaving



Figure 4. Subculture system for feed microalgae



and, from the start of the 1984/85 production season, all flask-flask sub-culturing was performed in an environment of very low bacterial population (i.e. a laminar flow cabinet, see Materials and Methods section A.3.1.1.2.i).

### 3.1.1 Preparation of Sterile Growth Medium.

#### 3.1.1.1 Static Cultures.

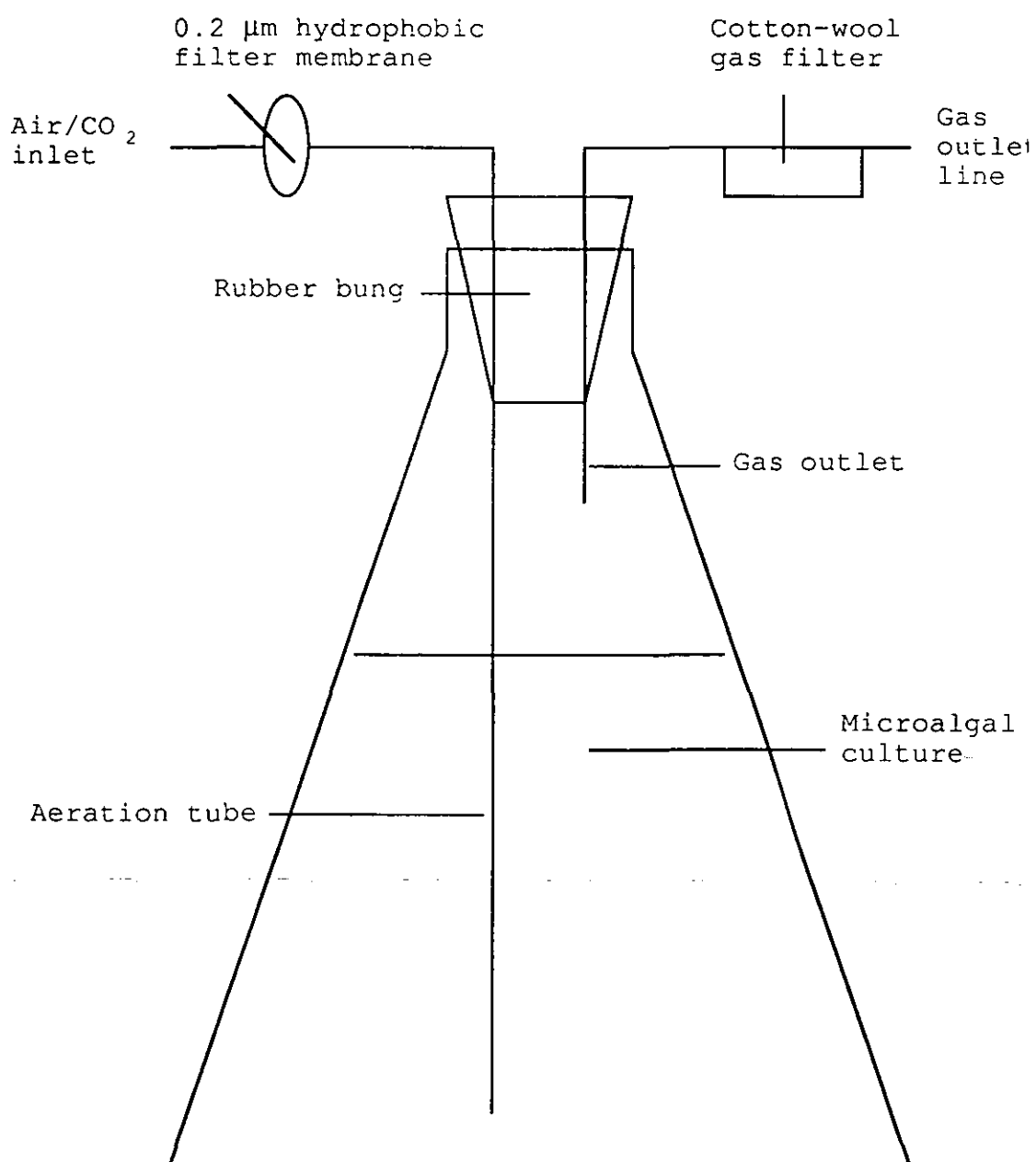
Flasks of sterile growth medium for the static (unaerated) culture of microalgae were prepared as follows.

Seawater (1  $\mu\text{m}$  filtered and pasteurised, 1983/84; 0.2  $\mu\text{m}$  membrane-filtered 1984/85 and 1985/86) was collected in a large (approximately 10 L) clean plastic container and nutrients added to produce Guillard's f/2 medium (Appendix 1). Growth medium was then dispensed into clean 250 or 500 mL Erlenmeyer flasks so that each flask was filled to  $1/2$ - $2/3$  capacity. Each flask was closed with a clean, dry bung (e.g. Steri-stopper, Heinz Heienz, West Germany) and the top  $1/3$  of the flask was capped with a double layer of aluminium foil. The foil cap was used to prevent dust and other particles from becoming trapped in the crevice between the bung and the lip of the flask. If this was not done these particles could fall into the culture when the bung was removed. The flasks were autoclaved immediately after filling and capping and afterward stored in a dry cupboard until required.

#### 3.1.1.2 Aerated cultures.

Large (5 L) flasks to be used for aerated microalgal cultures were filled to  $1/2$ - $2/3$  capacity with f/2 growth medium and assembled as shown in Figure 5. The assembled culture vessel was then loosely covered with an autoclave bag and autoclaved. Immediately upon removal from the

Figure 5. 5 L microalgae culture flask  
aeration system



autoclave the bag was closed tightly around the flask neck and the flask stored for later use.

i.) Flask-Flask Subculture Techniques.

Prior to the 1984/85 production season all microalgal subcultures were performed in a clean, covered enclosure designed to reduce the chances of airborne contamination of the cultures. However, poor aseptic technique during this procedure and the subsequent use of poorly maintained 0.2  $\mu\text{m}$  air filters for the 5 L flasks led to all flask cultures being contaminated by bacteria.

To remedy these problems, a simple, effective laminar-flow enclosure, in which flask-flask microalgal culture transfers could be performed, was designed and built by hatchery staff. The staff were instructed in aseptic transfer techniques and the type of 0.2  $\mu\text{m}$  air filters used for the 5 L flasks was changed to a one-piece, autoclavable unit (Millipore, Millex-50). These improvements resulted in the maintenance of axenic conditions in all flask cultures during 1984/85.

The procedure for performing flask-flask subculture of microalgal cultures was as follows.

- Remove the bung assemblies from both the parent culture and the sterile growth medium flasks and place them foil side down in the cabinet.

- Pass the rims of both flasks quickly through the flame of a Bunsen burner.

- Pour appropriate volume of inoculum from the parent stock to the new flask while taking care to avoid flask-flask contact and leaving at

least 1 mL of parent culture in the original flask after the last transfer is made. This small residual volume was used for subsequent bacteriological examination of the microalgal cultures.

- Flame the flask rims and bung assemblies and replace the bungs on their respective flasks.

## ii.) Culture Conditions.

Immediately after subculture the newly inoculated flasks were transferred to the culture room which was maintained at 20-26°C. Here they were put on metal racks which had about 90% open area. The racks held the flasks approximately 5 cm above a bank of 2 x 40 W White fluorescent light tubes.

Static cultures were swirled by hand once/day while 5 L cultures were connected to an air/CO<sub>2</sub> source. This supplied the cultures with a constant flow of air supplemented with CO<sub>2</sub> (C.I.G. food grade) to a final estimated concentration of 4% (v/v). The gas mixture was filtered prior to addition to the culture flask by passing it across a 0.2 µm air filter (Figure 5). Aerating the cultures in this way provided adequate water movement to keep the microalgal cells suspended in the water column while providing extra carbon dioxide to enhance the growth of the cells.

The flask cultures were incubated in this fashion for 4-10 days, until the hatchery staff needed them for further subcultures.

## 3.2 Culture of Microalgae in 500 L Bags.

Cultures of microalgae to be fed directly to oyster larvae were grown in 500 L transparent plastic bags (Renown and Pearlite, Melbourne) and harvested on a semi-continuous basis. These bags were produced by a heat-

extrusion method and were supplied in a fully sealed state. (i.e. the inside surface of each bag was sterile).

When filled, the bags had dimensions of 1.7 m (high) x 0.85 m (wide) x 0.42 m (deep). Filled bags were supported by an external wire frame cage and were placed beside a bank of 40-80 W white fluorescent light tubes which supplied continuous light with intensity  $0.52-1.10 \times 10^6$  quanta  $\text{sec}^{-1} \text{cm}^{-2}$  (Biospherical Instruments Inc., QSL-100) to the external surface of the bag close to the light source.

### 3.2.1 Preparation of Nutrient Solution.

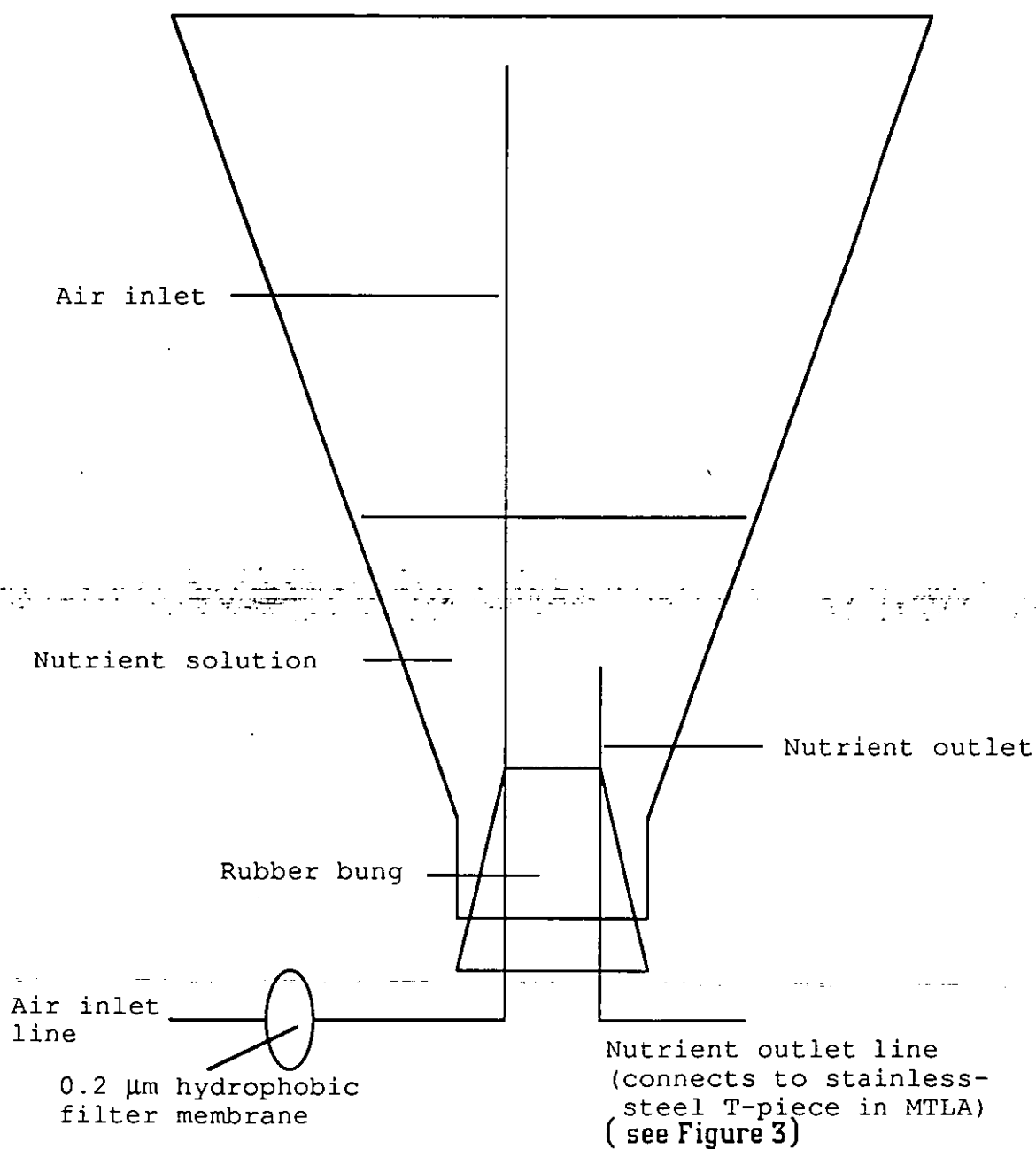
Nutrients for use in the growth medium for 500 L cultures of microalgae were prepared as described in Appendix 1. It was necessary to add the nutrients to the growth medium downstream of the final  $0.2 \mu\text{m}$  filter cartridge as the nutrient solutions contained fine crystalline precipitates. These crystals would have either blocked or destroyed the filter membrane.

Two litres of the final nutrient mix were placed in a clean 5 L Erlenmeyer flask and the complete flask assembly (Figure 6) autoclaved. Following sterilisation the complete unit was inverted and placed in a holder above the usual position of the T-piece in MTLA.

After the  $0.2 \mu\text{m}$  membrane-filtration unit had been assembled each day the foil caps covering the free end of the nutrient delivery line and the perpendicular arm of the T-piece were removed, the exposed ends sprayed with methylated-spirits and the hose pushed over the stainless-steel to form an airtight seal.

Nutrients could then be introduced into MTLA by opening the clamp valve in the nutrient line. Air entering the nutrient flask to replace the outflowing nutrient solution was filtered prior to entering the flask by passing across a  $0.2 \mu\text{m}$  air filter.

Figure 6. System for dispensing nutrients to 500 L microalgal cultures



When a second nutrient dispenser was needed (e.g. if the first flask was empty) it was placed in a second holder adjacent to the original flask. The delivery line from the first flask was disconnected from the T-piece, the exposed end of the new nutrient delivery line sprayed with methylated-spirits and connected to the T-piece.

When a nutrient flask was empty or was not to be used again on a specific day, it was dismantled, cleaned with hot (approximately 60°C) fresh water and allowed to air dry until next required (usually the next day).

### 3.2.2 Preparation of 500 L Microalgal Culture Bags.

To prepare a 500 L bag for filling, a new, unopened bag was placed within a wire frame and aligned so no creases would form during filling. A top corner of the bag was disinfected with methylated-spirits and cut off, using scissors which had also been disinfected with methylated-spirits, to leave a hole approximately 1.5 cm wide. The syringe-cylinder end of MTLB was removed from its autoclave bag, sprayed with methylated-spirits and inserted into the hole. The bag was taped firmly (airtight) around the syringe cylinder. An air line was connected to the free end of the 0.2  $\mu\text{m}$  air filter and the bag inflated with 0.2  $\mu\text{m}$  membrane-filtered air.

When the bag was full of air the Nalgene hose of MTLB was removed, the syringe cylinder sprayed with methylated-spirits and the free end of MTLA inserted (airtight) into the cylinder. An area on the top of the bag was sprayed with methylated-spirits and a small (approximately 1 cm) cut made in the bag to allow air to escape while the bag was filling with growth medium. Immediately the cut was made 0.2  $\mu\text{m}$  membrane-filtered seawater was pumped into the bag via MTLA.



When the bag was about  $1/2$  full the pump was stopped and the appropriate volume of sterile nutrient solution was fed into MTLA from the nutrient dispenser (Materials and Methods section A.3.2.1). The pump was restarted and the bag filled. The air escape hole was taped shut (airtight) immediately after the bag was filled with microalgal growth medium.

### 3.2.3 Inoculation of 500 L Bags with Microalgal Starter Culture.

All 500 L bags were inoculated with microalgae directly from 5 L aerated cultures. Inoculation occurred immediately a bag had been filled with microalgal growth medium. One 5 L flask culture was used to inoculate one 500 L bag.

A 5 L flask containing a healthy (as determined by hatchery staff) culture of microalgae was placed on a stable platform near the bag to be inoculated. The filter assembly on the gas inlet tube on the flask (Figure 7) was removed and the newly exposed end of the glass aeration tube disinfected with methylated-spirits. The thin end of MTLC was removed from its autoclave bag and pushed over the glass tube to form an airtight seal. The end of MTLA was removed from the syringe cylinder in the top of the bag and the free end (silicon hose) of MTLC inserted into the syringe cylinder. An air line was attached to the cotton wool gas filter assembly on the flask and air pumped into the flask through the original gas outlet line forcing the microalgal culture to flow out of the flask and into the 500 L bag.

When inoculation was completed the thin Nalgene hose was disconnected from the nipple of the syringe cylinder of MTLC and was replaced by a short (approximately 30 cm) length of sterile (autoclaved) thin Nalgene hose. This new hose acted as the air outlet line from the 500 L bag. The end of this hose was permanently immersed in a 10% (w/v)

HCl solution which acted to reduce the chance of cross-contamination of bag cultures via aerosols escaping from the bags.

Aeration was provided to the bags through MTLTD. The free end of the 0.2  $\mu\text{m}$  air filter was connected to the air/ $\text{CO}_2$  supply (Materials and Methods section A.3.1.1.2.ii). The Pasteur pipette was gently heated with a gas flame and quickly inserted through the bag wall as near as possible to the bottom of the bag. This formed a watertight seal. The inflowing gas mixture provided complete mixing of the microalgal culture.

A diagram of an operational 500 L bag is shown in Figure 8.

#### 3.2.4 Harvesting Feed Microalgae from 500 L Bags.

When the density of microalgal cells in 500 L bag cultures was determined by hatchery staff to be adequate for harvesting as food for oyster larvae, the appropriate bags were prepared for harvesting and subsequent refilling with growth medium. Following this, each culture could be harvested and refilled on a semi-continuous basis until the culture was considered no longer suitable for use as food for oyster larvae. Decisions to discard a culture were made for a variety of management and/or biological reasons.

##### 3.2.4.1 Installing the Harvest/Refill Valve (MTLF).

To allow a bag culture to be harvested and the bag to be refilled with microalgal growth medium a harvest/refill point was needed. To achieve this a sterile MTLF unit was aseptically inserted into the side of the appropriate bag as follows.

An area approximately half-way up the side of the bag was sprayed with methylated-spirits. A metal spike with a maximum diameter about 2 mm less than that of the syringe cylinder on the MTLF unit was heated with a gas flame and immediately pushed through the disinfected area of

Figure 7. System for inoculating 500 L bags with 5 L microalgal starter culture.

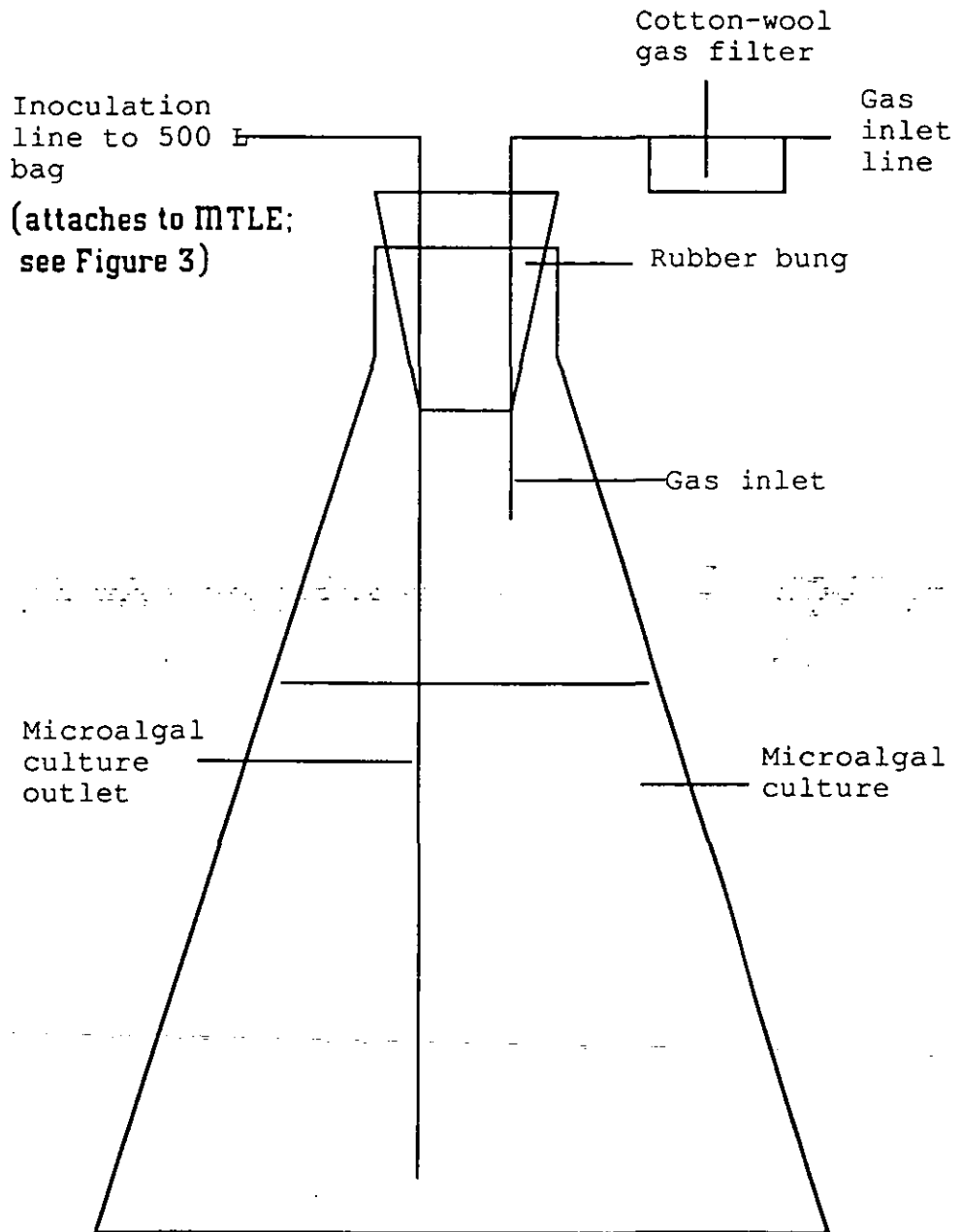
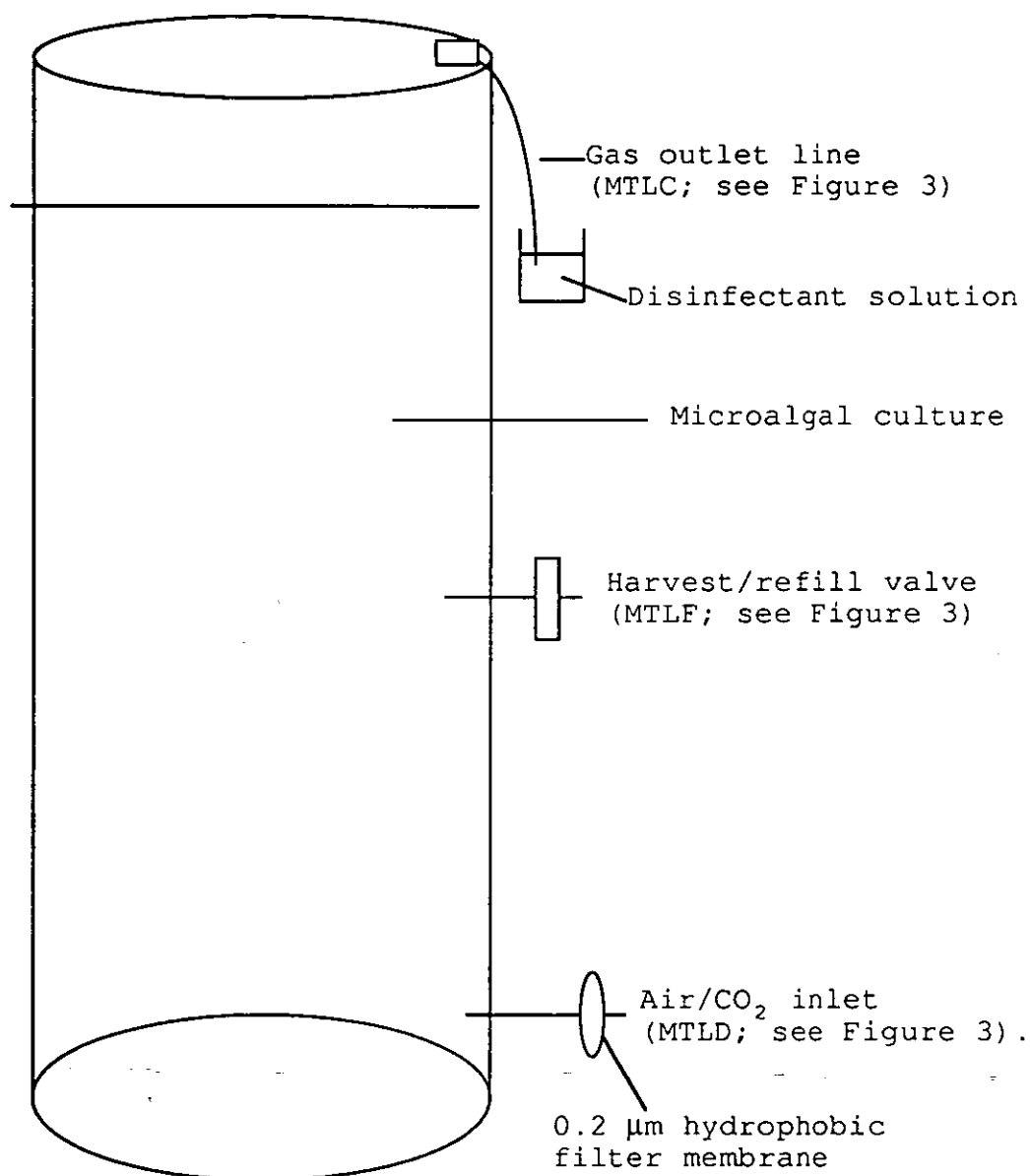


Figure 8. Operational 500 L microalgae culture bag



the bag wall, forming a watertight seal. A sterile MTLF unit was removed from its autoclave bag and sprayed with methylated-spirits. The spike was removed from the bag wall and the syringe cylinder end of the MTLF unit immediately pushed into the hole, forming a watertight seal.

All future passages of liquid out of and into the bag were performed using this unit.

Harvest/refill valves were usually only inserted into the culture bags on the day of the first harvest from that bag. However, to assist with bacteriological monitoring, valves were inserted into selected bags immediately after they were filled with uninoculated growth medium.

#### 3.2.4.2 Harvesting the Microalgal Cultures.

Microalgae was harvested from the 500 L bags as required using sterile (autoclaved) MTLG units to transfer the culture to a collecting tank. One end of a MTLG unit was removed from its autoclave bag and sprayed with methylated-spirits. The free end of the harvest/refill valve (MTLF) was sprayed with methylated-spirits and the harvest line (MTLG) attached to the valve. The other end of the harvest line was placed in a clean 700 L fibreglass collecting tank and the required volume of microalgal culture harvested from the bag into the tank.

When a sufficient volume of microalgal culture had been collected the harvest line was removed for cleaning and autoclaving and the harvest/refill valve disinfected with methylated-spirits.

#### 3.2.4.3 Delivering Feed Microalgae to Larvae.

When the desired volume and mix of the various microalgal cultures had been harvested into the collection tank it was pumped to the appropriate larvae tanks along 25 mm bore Nalgene (food grade) delivery line.

#### 3.2.4.4 Cleaning the Microalgae Collection Tank and Delivery Lines.

Immediately after all harvested microalgae had been pumped to the larvae tanks the fibreglass tank was rinsed with fresh water, scrubbed by hand with a soft nylon brush and rinsed again with fresh water. The tank was filled with hot (approximately 60°C) fresh water which was then pumped along all the microalgal delivery lines used that day. When emptied the tank was inverted and air dried until next required (usually next day).

Every two weeks during the production season the fibreglass tank, delivery lines and pump were disinfected by filling them with a commercial disinfectant (Safsol) prepared at a final concentration of 100 ppm free chlorine. The solution was left to stand in the tank, pump and delivery lines for one hour. After this it was drained and the delivery system rinsed with 0.2 µm membrane-filtered seawater and air dried before next use.

#### 3.2.4.5 Refilling the 500 L Bags with Microalgal Growth Medium.

Following the harvest of a volume of microalgal culture from a 500 L bag the bag was refilled with fresh 0.2 µm membrane-filtered growth medium through the harvest/refill valve. The exposed valve hose-tail and the free end of MTLA were disinfected with methylated-spirits and joined to form a watertight seal. The appropriate volume of nutrient solution was introduced into MTLA from the nutrient dispenser and the pump used to push the fresh seawater through the 0.2 µm membrane-filtration system started. The harvest/refill valve was immediately opened and the bag allowed to fill. When the bag was full the valve was closed, the pump stopped and MTLA removed from the valve hose-tail. The sequence of starting and stopping the pump and opening and closing the harvest/refill

valve was important to prevent any bacteria from within the 500 L bags contaminating the end of MTLA.

Never-the-less the hose-tail and the free end of MTLA were always disinfected with methylated-spirits before MTLA was used to add growth medium to the next bag. It should be noted here that any new 500 L bags were always filled before MTLA was used to top up harvested cultures. This was done to reduce the chances of contaminating the new cultures by the transfer of bacteria on MTLA.

#### 4.0 Larval Culture and Spat Production.

Adult *C. gigas* broodstock were regularly spawned at the hatchery during each production season. Production was divided into 4 main areas: broodstock conditioning; induction of spawning; fertilisation and incubation; and larval rearing. The methods employed for these facets of spat production are detailed below.

##### 4.1 Broodstock Conditioning.

Adult oysters (1-3 years old) for use as broodstock were selected from commercial oyster farms throughout Tasmania. The broodstock were collected 2-3 months before the start of each spawning season and kept under controlled conditions at the hatchery.

##### 4.1.1 Water Temperature.

Ambient seawater temperatures were usually between 12-15°C when the broodstock were collected. After transfer to the hatchery the broodstock oysters were placed in shallow (1 m x 2 m x 15 cm deep) conditioning tanks and supplied with fresh filtered seawater at a constant flow rate of approximately 1 L hr<sup>-1</sup> oyster<sup>-1</sup>. The temperature of the water being supplied to the conditioning tanks was raised over a period of

6-8 days from ambient to the final conditioning temperature of 19°C. The oysters were held at this temperature until required for spawning.

#### 4.1.2 Provision of Nutrients.

Broodstock oysters were fed with the same species of microalgae as used as food for the larvae. The microalgae were cultured in 1 µm depth-filtered seawater in open fibreglass tanks sited outside the hatchery, under ambient lighting and temperature conditions. These cultures were continuously pumped into the conditioning tanks to yield a final concentration of 20,000-50,000 microalgal cells mL<sup>-1</sup>.

#### 4.2 Spawning Induction.

On the night prior to a planned spawning, broodstock (usually 50-90 animals) were removed from the conditioning tanks, scrubbed clean with a soft nylon brush and placed in a shallow (1 m x 1.5 m x 10 cm deep) spawning tray. They were left out of water overnight (approximately 14 hr) at a controlled temperature of 19 ± 1°C.

The following morning the spawning tray was filled with fresh filtered (1 µm) seawater at 20°C. During the next 2 hr the temperature of the water in the spawning tray was slowly raised to 25 ± 1°C. Water temperature was maintained at this level for the duration of the spawning session. Gamete release usually commenced between 1-3 hr after the addition of the 20°C water to the spawning tray.

As soon as each oyster started spawning its sex was determined by means of the physical characteristics of the released gametes. The spawning oyster was then removed from the spawning tray and placed in a separate tank containing isothermal water. Spawning broodstock were separated according to sex to achieve a greater level of control over the



fertilisation process. The oysters continued to spawn normally in the separate containers.

#### 4.3 Fertilisation and Incubation

When sufficient gametes had been obtained the broodstock were removed from the secondary spawning containers and the eggs transferred to a clean 70 L container. Fresh, filtered (10  $\mu\text{m}$ ) seawater was added to bring the total volume to 50 L and the final temperature to 24-25<sup>a</sup>°C. The eggs were suspended evenly throughout the water column in this tank by mixing with a clean plastic paddle and a 1 mL aliquot of the even egg suspension taken. The eggs in the sample were examined by light microscopy in a Sedgwick-Rafter type chamber to determine the fertilisation rate. The eggs in this sample were also counted in order to determine the total number of eggs in the 70 L container. If the fertilisation rate was < 90% a 500 mL sample of fresh sperm suspension was added to the 50 L tank and thoroughly mixed with the eggs. The eggs were left to stand for another half hour when the fertilisation rate was rechecked. At this stage the fertilisation rate was invariably > 95%.

Fertilised eggs were transferred for incubation to 7000 or 10,000 L cylindrical larvae tanks which had been previously filled with fresh, filtered (10  $\mu\text{m}$ ) seawater at 24-25°C. The eggs were distributed amongst the tanks to give a final density of about 30 mL<sup>-1</sup> (Table 8).

#### 4.4 Larval Rearing.

The process of larvae rearing involved maintaining the larvae in hygienic conditions at the appropriate temperature, adjusting the density of the larvae in the larvae tanks and feeding them sufficient quantities of the appropriate diet of microalgae.

<sup>a</sup>. At this stage approximately 250 mL of an even mixture of sperm filled seawater from each spawning male was added to the egg suspension. The egg/sperm suspension was then stirred using a clean plastic paddle, to ensure thorough mixing, and left to stand for approximately 30 minutes.

Table 8. Husbandry guidelines for rearing *C. gigas* at 25-28°C.

Approximate larval age (days)	Size range of larvae (µm)	Approximate larval density (mL <sup>-1</sup> )	Approximate vol. feed microalgae <sup>a</sup> added 1000 L <sup>-1</sup> larval culture medium (L)
0	eggs	30 <sup>b</sup>	0
1, 2	80- 88	16	15-20
3, 4	89-105	10	20-25
5, 6	106-119	7	25-30
7, 8	120-139	5.3	30-35
9, 10	140-167	4.2	30-35
11, 12	168-211	3.7	35-40
13, 14	212-254	2.6	35-40
15, 16	255-299	2.0	35-40
17, 18	300-316	1.8	35-40
19+	>316	1.5	40-45

<sup>a</sup>: The daily diet of mixed clones is shown in Table 9.

<sup>b</sup>: Fertilised eggs.

#### 4.4.1 Changing the Growth Medium.

To maintain hygienic conditions in the larval tanks, the larval growth medium needed to be changed, and the tanks cleaned (see Materials and Methods section A.4.4.3), every one or two days. If this was not done, accumulation of larval metabolites and senescent microalgal cells in the tanks would lead to a rapid deterioration of water quality and the subsequent death of larvae.

The larvae were reared in seawater, filtered to 25  $\mu\text{m}$  and heated to between 24-28°C as described in Materials and Methods section A.1.2.1.

The first water change was carried out approximately 24 hours post-fertilisation. At this stage all the fertilised eggs had hatched and the larvae developed to the veliger (D-shaped) stage. If the water was changed before the larvae had reached this stage (i.e. while they were still at the trochophore stage) many of the larvae would have been damaged and killed. Trochophore oyster larvae can not withstand the physical rigours of being collected onto a sieve as they do not have fully developed larval shells and have not undergone torsion (MacBride 1914, Loosanoff and Davis 1963).

Subsequent water changes were carried out every one or two days, depending on the workload in the hatchery and the growth and survival rate of a specific batch of larvae. If the hatchery staff considered that the larvae were unhealthy, by either morphological or behavioural signs, they changed the culture medium of that batch of larvae every day.

Water changes were achieved by draining the larvae tanks through nylon sieves of specific mesh size. During this operation the sieves were immersed in a water bath to avoid damaging the larvae. Abnormal larvae and undeveloped eggs were smaller than normally developing larvae. They could therefore be separated from the normally developing larvae by

collecting the larvae on a series of sieves of sequentially decreasing mesh size.

Larvae which had been collected on a sieve were washed with fresh, filtered (10  $\mu$ m) seawater while still on the sieve and transferred to a clean 20 L container. Seawater was added to this container to bring the total volume to 10 L. The larvae were mixed to evenly distribute them in the water column and 2 x 0.1 mL aliquots of the larval suspension were obtained. Active larvae were examined by light microscopy for swimming activity, morphology and size. The larvae in the aliquots were then killed by adding a drop of an aqueous sodium hypochlorite solution (12.5% free chlorine; Protect-a-Clean, Tasmania) and counted to estimate of the total number of larvae in the 10 L container. If the counts from the 2 aliquots were within 10% of each other, the average was taken. If the counts obtained from the 2 aliquots varied from each other by more than 10% another 2 samples were counted. The average of all 4 counts was then used to calculate the total number of larvae in that batch.

Larvae were culled (if necessary) by discarding all or some of the larvae collected on the finest (smallest) sieve used for a water change. The remaining larger larvae were resuspended at the required density (Table 8), in a clean larvae tank which had been previously filled with fresh, filtered seawater at the appropriate temperature.

#### 4.4.2 Provision of Nutrients.

The volume and composition of microalgal cultures to be fed to the larvae were determined by the hatchery staff based on a number of criteria, including: the number of larvae in the larvae tanks; the age and size of the larvae; the amount of uneaten food in the larvae tanks; the volume of microalgal cultures available for feeding.

The larvae were fed a diet of mixed microalgae which were grown, harvested and delivered to the larvae tanks as described in Materials and Methods section A.3.2. The composition of this diet varied with age (Table 9).

#### 4.4.3 Cleaning the Larval Tanks.

When a larvae tank had been emptied it was cleaned by scrubbing the walls and floor with a soft nylon brush using a commercial disinfectant (Safsol) prepared to a final concentration of 100 ppm free chlorine. The disinfected tank was rinsed with fresh, filtered (25  $\mu$ m) seawater and air dried for at least 8 hr before refilling.

### B. Bacteriological Cultivation.

#### 1.0 Bacteriological Culture Media and Conditions.

All samples collected from the hatchery and seawater supply system were cultivated on Sea Water Agar with Vitamins (SWAV, Appendix 2.3), Thiosulphate-Citrate-Bile salt-Sucrose agar (TCBS, Appendix 2.4) or Plate Count Agar (PCA, Appendix 2.5) at 20-22°C. The total number of viable heterotrophic bacteria was counted on SWAV and PCA after 7-10 days incubation and presumptive *Vibrio* colonies were counted after 2-3 days incubation on TCBS agar.

Table 9. Approximate % (v/v) composition of microalgal clones in mixed diets fed daily to *C. gigas* larvae in successive production seasons.

Production season	Clone <sup>a</sup>						
	T. Iso	C. cal	3C	3H	Dun	Pav	Tet
1983/84	% of diet	50-100	20-50	5-35	nf <sup>b</sup>	10-20	nf
	age of larvae fed	1d-r <sup>a</sup>	1d-r	2d-r		11d-r	
1984/85	% of diet	30-50	10-70	10-60	10-25	5-20	nf
	age of larvae fed	1d-r	1d-15d	2d-r	5d-r	11d-r	
1985/86	% of diet	20-40	5-15	5-25	15-25	5-20	20-40
	age of larvae fed	1d-r	1d-7d	1d-r	3d-r	10d-r	1d-r

<sup>a</sup>: see Abbreviations, page 4.

<sup>b</sup>: not fed.

<sup>c</sup>: ready-to-set larvae.

## 1.1 Liquids.

### 1.1.1 Sampling Techniques.

#### 1.1.1.1 Seawater.

Samples of seawater were obtained from the primary filter tower, the pre-filtration system in the algal culture room and the 0.2  $\mu\text{m}$  membrane-filtration system after allowing a stream of seawater from the appropriate source to run to waste for about 30 seconds and then into a 100 mL sterile (autoclaved) screw-top bottle.

Samples obtained from oceanic water near the intake line, the settlement tank, the header tank and larvae tanks were collected with a sterile 50 mL syringe (Terumo). The contents of the syringe were immediately transferred aseptically into a sterile, screw-top glass bottle.

#### 1.1.1.2 500 L Microalgal Cultures.

Samples of microalgal cultures from selected 500 L bags were obtained through the harvest/refill valves. Each valve was disinfected with methylated spirits and allowed to air dry for about 1 minute. The valve was fully opened and a stream of culture allowed to run to waste for about 5 seconds and then into a sterile screw-top glass bottle.

#### 1.1.1.3 Flask Microalgal Cultures.

Microalgae from the culture flasks were obtained directly from the flasks with a sterile 1.0 mL pipette and immediately diluted in 9.0 mL of sterile (autoclaved) seawater (see Materials and Methods section B.1.1.2.2).

### 1.1.2 Bacteriological Cultivation Techniques.

All samples were prepared and inoculated onto agar plates within 30 minutes of sampling.

#### 1.1.2.1 0.2 $\mu\text{m}$ Membrane-Filtered Seawater.

Bacteria in 100 mL samples of 0.2  $\mu\text{m}$  membrane-filtered seawater were collected on sterile 0.1 or 0.2  $\mu\text{m}$  membrane discs (Millipore) at low vacuum pressure (approximately 30 kPa). The discs were placed on agar plates and incubated and counted as described in Materials and Methods section B.1.0.

#### 1.1.2.2 Other Liquid Samples.

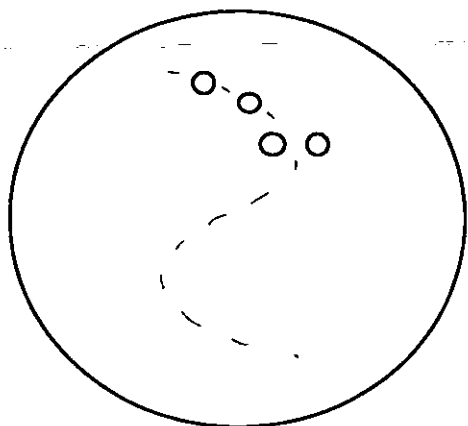
Viable bacteria in samples of microalgal cultures and seawater were enumerated by inoculating (spread plate) 0.1 mL aliquots of serial 10-fold dilutions (using sterile seawater as the diluent) of the cultures, in duplicate, onto SWAV and TCBS.

### 1.2 Surfaces.

In addition to determining the number of viable bacteria in various liquid samples in and around the hatchery, it was important to assess (semi-quantitatively) the level of bacterial contamination of hatchery fomites, especially the internal surfaces of plumbing items used to transfer microalgal growth medium to the 500 L bag cultures. Monitoring was carried out to assess the efficacy of various disinfection regimes and to obtain an indication of the rate of bacterial colonisation of the disinfected surfaces.

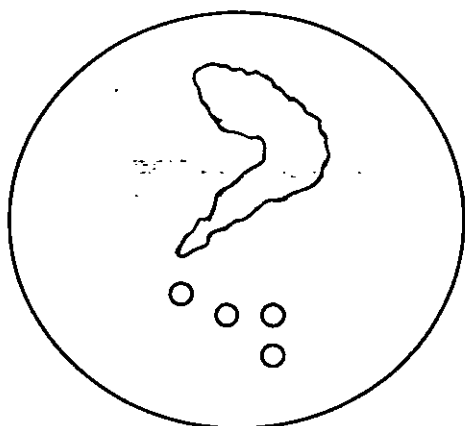
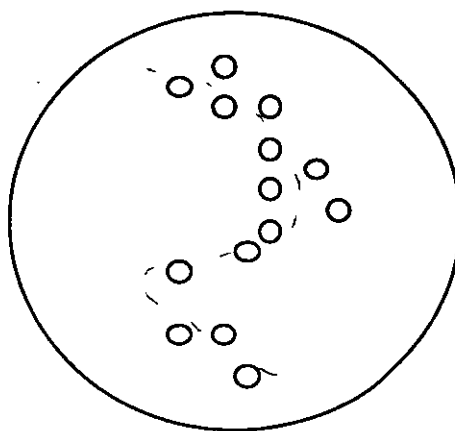


Figure 9. Semi-quantitative scoring scheme  
for swab plates



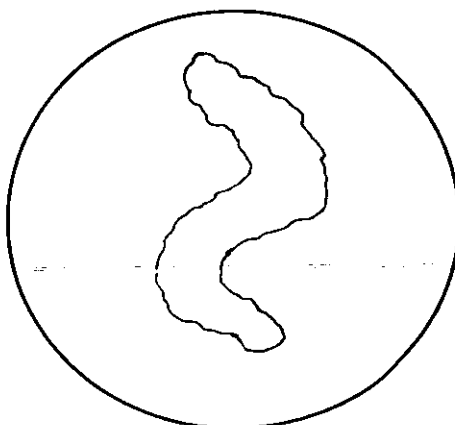
+ (few discrete colonies)

++ (many discrete colonies)



+++ (some confluent growth  
many discrete colonies)

++++ (all confluent growth)



Bacteria on surfaces were sampled by swabbing approximately 16 cm<sup>2</sup> of the surface with a sterile (autoclaved) swab stick (e.g. Cotton buds, Johnston and Johnston) and immediately inoculating onto an agar plate in a zig-zag pattern (Figure 9). Duplicate areas were swabbed at each sample site so both SWAV and TCBS plates could be inoculated

If the surface to be tested was dry, the swab stick was dipped into sterile (autoclaved) seawater before the surface was swabbed. A new container of sterile seawater was used for each sample and swab stick.

A semi-qualitative measure of levels of bacterial contamination of surfaces was derived using this method of sampling. Increasing levels of bacterial contamination on the tested surfaces resulted, after incubation, in bacterial growth on the agar plates ranging from 0 to ++++ (Figure 9). Variations in the degree of contamination of most surfaces could be monitored using this scoring scheme.

### 1.3 Oyster Larvae.

The total number of viable bacteria associated with sick and healthy larvae of different sizes was examined. Larvae were collected in a fine mesh sieve and washed with 3 x 1 L sterile seawater. The larvae were transferred to a second screen, which had been disinfected with methylated-spirits and thoroughly rinsed with sterile seawater immediately prior to receiving the larvae. The larvae were again rinsed with 3 x 1 L sterile seawater and suspended in a small (10-20 mL) volume of sterile seawater.

A 1.0 mL aliquot of the larval suspension was transferred into a sterile (autoclaved) glass grinding tube (Corningware) and the larvae ground by hand to produce a homogenate containing fragments no larger than 5 µm at their greatest diameter (determined by light microscopy). The total counts of viable heterotrophs and presumptive *Vibrio* spp. in the

homogenate were determined as for liquid samples (Materials and Methods section B.1.1.2.2).

The concentration of washed larvae in the homogenate was determined by taking the mean count of larvae in 3 x 0.1 mL samples of the suspension of washed larvae.

#### 1.4 Eggs.

The level of bacterial colonisation of freshly released fertilised and unfertilised eggs was also examined. Eggs were collected on a fine mesh sieve and washed in the same fashion as larvae (Materials and Methods section B.1.3). Washed eggs were suspended in a small volume (10-20 mL) of sterile seawater and 0.1 mL aliquots of serial 10-fold dilutions of this suspension inoculated (spread-plate) onto SWAV and TCBS.

The concentration of eggs in the original suspension was determined in the same way as described in Materials and Methods section B.1.3.

#### 1.5 Air.

Routine testing of the efficacy of the laminar-flow unit was carried out by placing freshly prepared SWAV and PCA plates in the cabinet while microalgal sub-culturing was being performed. The lids were removed from the petri-dishes and the growth media exposed to the air in the cabinet for 10-15 minutes. The lids were replaced and the plates incubated as described in Materials and Methods section B.1.0.

Control tests were performed by repeating the above procedure in an open area on the work bench adjacent to the laminar-flow unit.

#### 2.0 Statistical Analysis.

Comparisons of means were performed using Students t-test as described in Clarke (1969).

## C. Microscopy.

### 1.0 Scanning Electron Microscopy.

Samples of microalgal cultures and freshly released eggs (fertilised and unfertilised) were examined for the presence of surface associated bacteria by scanning electron microscopy (SEM).

Suspensions of eggs or microalgae were mixed with an equal volume of combined aldehyde fixative (Appendix 3.3). Drops of this suspension were then placed on a poly-lysine coated (Marchant and Thomas 1983) glass cover slip and the cells allowed to settle onto the glass over several hours.

The fixed cells were gently rinsed with an aqueous 1 % (w/v) NaCl solution and immediately dehydrated as follows:

Aqueous ethanol % (v/v)	NaCl % (w/v)	Acetone % (v/v)	Immersion time (minutes)
25	0.5	-	15
50	0.1	-	15
75	0.1	-	15
95	0.1	-	15
100 (dry)	-	-	15
100 (dry)	-	-	30
-	-	100 (dry)	15
-	-	100 (dry)	15

The dehydrated specimens were immediately critical point dried (Polaron E3000), attached to aluminium SEM stubs (Ladd) using copper

print (G.C. Electronics), sputter coated with approximately 20 nm gold (Balzers Union) and examined in a Philips 505 scanning electron microscope at 15 kV. Micrographs were recorded on Ilford FP4 film, ASA 100.

## 2.0 Light Microscopy.

In an attempt to decrease the time taken to establish the level of bacteria in microalgal cultures, a direct (light microscopy) method of counting the bacteria, based on that used by Bitton *et al.* (1983), was tested.

All glassware used for this experiment was washed thoroughly in fresh (tap) water, rinsed twice with absolute ethanol (Ajax) to remove any particulate matter from the glass surface and autoclaved before use.

Duplicate samples of microalgal culture were collected from a 500 L bag into sterile (autoclaved) screw-top glass jars. The contents of one jar were passed across a sterile 3  $\mu\text{m}$  membrane disc (Millipore) and the filtrate collected in a sterile glass container. A 1.0 mL aliquot was aseptically taken from each of one of the filtered and unfiltered samples and 10-fold serial dilutions of the aliquots inoculated onto SWAV plates as described in Materials and Methods section B.1.1.2.2.

The second 1.0 mL sample of each of the filtered and unfiltered microalgal cultures was mixed with an equal volume of mixed aldehyde solution (Appendix 3.3) and passed across a 0.1  $\mu\text{m}$  filter disc (Millipore). The microorganisms were fixed on the discs by drying in an oven at 95-100°C for 5 minutes. The filter membrane was cleared by floating the disc, organisms up, on the surface of immersion oil (518C; Zeiss, West Germany). Stain [1% (w/v) Malachite green] was added to cover the surface of the filter disc and allowed to stand for 5 minutes. Excess stain was gently blotted off and a drop of immersion oil added to the area to be

examined. A microscope coverslip was placed over the oil and the sample examined under oil immersion at a magnification of 400 x.

Microalgae (unfiltered) and bacteria (filtered) were counted in 20 squares of an ocular lens grid system.

## RESULTS

### A. 1983/84 Production Season.

#### 1.0 Introduction.

Preliminary data implicating bacteria in microalgal cultures as the cause of larval mortality at the hatchery were first compiled during the 1983/84 production season by Dr. C.D. Garland (Dept. of Agricultural Science, University of Tasmania). Research during the 1983/84 production season, prompted by the economically significant larval mortalities then being experienced by the hatchery, indicated the need for a comprehensive bacteriological study to identify the source of the suspected pathogens and to rectify the problem.

#### 2.0 Preliminary results.

Results from Dr. Garlands' work (Table 10; Figure 10) and information derived from hatchery records are presented in this chapter so that the improvements implemented during the 1984/85 and 1985/86 production seasons can be appreciated.

Macroscopically, losses of larvae were indicated as deposits of animals in discrete clumps on the bottom of the larval tanks. Microscopic examination of the deposits revealed larvae with a range of abnormal features. Mildly- to moderately-affected larvae swam irregularly or not at all. The velum was often bald (deciliated), the gut was pale, indicating poor feeding, and the valve edge was often misshapen. Severely affected animals exhibited necrosis or dissolution of internal organs. These signs were typical of pathogenesis types 2 and 3 described in Elston and Liebovitz (1980).

Table 10. Bacteriological data on cultures of feed microalgae and other specimens related to the rearing of larval oysters in season 1983/84.

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- Viable bacteria (SWAV; mean  $\pm$  sd  $\log_{10}$  units  $\text{mL}^{-1}$ ) in feed microalgal cultures:

<i>Isochrysis galbana</i> , (Clone T.Iso)	6.3 $\pm$ 0.7 (n = 119)
<i>Chaetoceros calcitrans</i>	7.2 $\pm$ 0.4 (n = 16)
<i>Dunaliella tertiolecta</i>	6.5 $\pm$ 0.8 (n = 22)
<i>Chroomonas salina</i> 3C	6.3 $\pm$ 1.1 (n = 7)
all feed microalgal cultures	6.4 $\pm$ 0.8 (n = 164)

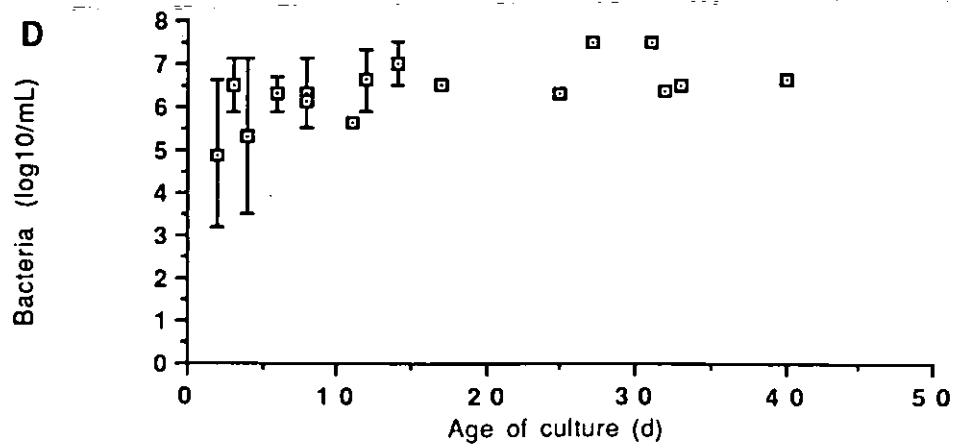
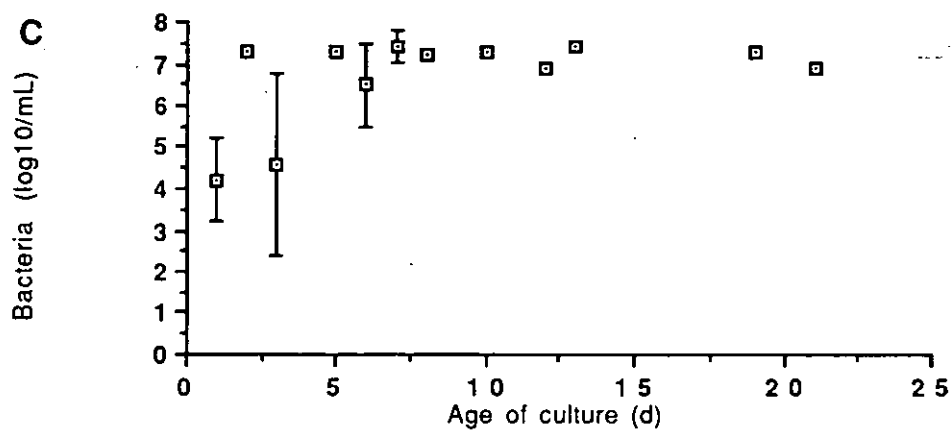
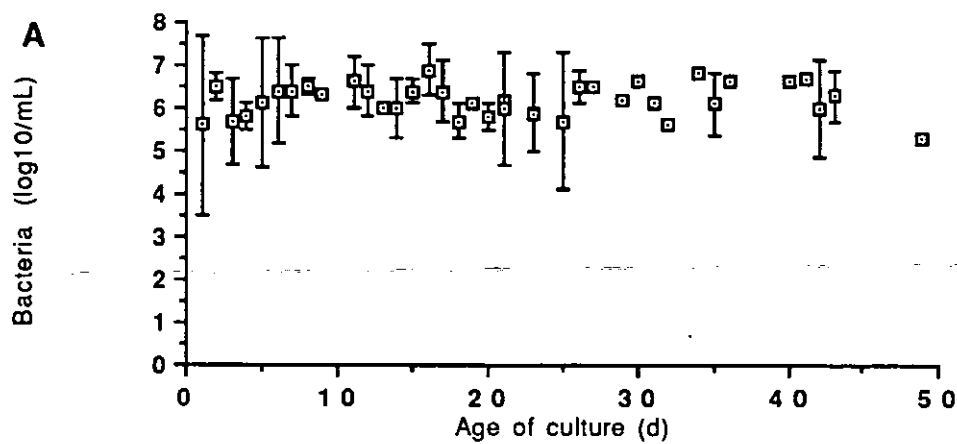
- growth medium for feed microalgae (pre-inoculation) 4.1 $\pm$ 0.8 (n = 8)
  - % feed microalgal cultures with  $> \log_{10}$  6.3 bacteria  $\text{mL}^{-1}$  55.6
  - Base for microalgal growth medium pasteurised-cooled seawater
  - Bacterial quality of 5L microalgal starter culture non-axenic
-



Figure 10. Viable (SWAV) bacteria in 500 L semi-continuously harvested microalgal cultures fed to larvae in 1983/84. Points with bar markers represent mean  $\pm$  sd value on each day of sampling. Single points indicate counts for which only one value was recorded.

Key.

- A. *Isochrysis* sp. (T. Iso)
- B. *Chroomonas salina*
- C. *Chaetoceros calcitrans*
- D. *Dunaliella tertiolecta*



These signs of disease in larval oysters strongly suggested that marine bacteria were responsible for the mortalities. Therefore various specimens were examined bacteriologically. The broodstock, intake seawater, and hatchery fomites were found to be colonised by low levels of bacteria and were not considered responsible for shedding high doses of microorganisms into the hatchery. However, the feed microalgae (microalgal cultures grown in the 500 L bags) were found to contain high levels of bacteria.

Bacteriological results collected during the 1983/84 production season indicated that the cultures of microalgae grown as food for the larval oysters were responsible for outbreaks of bacterial disease of larvae. Concentrated samples of feed microalgae were examined by SEM using methods which preserve bacteria on surfaces. Bacteria were found associated with the surface of all microalgal clones. However, surface associated bacteria were seen in <sup>only</sup> 16 of 42 (38%) specimens studied by SEM. This suggested that the majority of bacteria in most cultures of feed microalgae were free-living. <sup>only</sup>

## 2.1 Microalgal Growth Medium.

Seawater used in the growth medium for feed microalgae during the 1983/84 season was first pasteurised, then cooled for up to 36 hours (as described in Materials and Methods section A.1.2.2.1). Immediately after pasteurisation, the seawater contained  $<1$  viable (SWAV) bacteria  $\text{mL}^{-1}$ . After partial cooling, for 8-10 hours in the pasteurisation tanks, and delivery along plastic and heat-exchange pipes from the tanks to the 500 L bags, the seawater contained  $\log_{10} 2.7 \pm 0.9$  bacteria  $\text{mL}^{-1}$ . This latter count was lowest immediately after the delivery tubes had been disinfected but always exceeded  $\log_{10} 1.48$  bacteria  $\text{mL}^{-1}$ . After cooling for a further 24 hours in the 500 L bags, post-pasteurised seawater contained  $\log_{10}$

$4.1 \pm 0.8$  bacteria  $\text{mL}^{-1}$  (Table 10). As shown in Table 10, one 5 L starter culture of non-axenic (i.e. one which had been inadvertently contaminated with bacteria) microalgae was inoculated into each 500 L bag containing the pasteurised-cooled seawater. Sterile (autoclaved) nutrients for microalgal growth were also added.

## 2.2 Harvest Regime and Bacterial Content of Feed Microalgae.

### 2.2.1 Harvest Regime.

Microalgae to be fed to oyster larvae were harvested from the 500 L bags on a semi-continuous basis (eg. approximately 100 L every two days) (Table 11). In 1983/84 *Isochrysis* sp. (T. Iso), fed to larvae throughout the growth cycle (Table 9), was the most commonly used clone. *C. calcitrans* was used less frequently, usually for larvae of  $< 200 \mu\text{m}$  diameter. *D. tertiolecta* was generally fed to animals  $> 130 \mu\text{m}$  size. *Ch. salina* 3C was fed sparingly throughout the larval cycle.

### 2.2.2 Bacteriological Results.

The total levels of viable (SWAV) bacteria found in the 4 microalgae clones, at harvest, used during this season are shown in Table 10. The highest mean count,  $\log_{10} 7.2$  units  $\text{mL}^{-1}$ , occurred in cultures of the centric diatom *C. calcitrans*. The 3 flagellates *Isochrysis* sp. (clone T. Iso), *Ch. salina* 3C and *D. tertiolecta* supported bacterial populations with a mean level of  $\log_{10} 6.3$  units  $\text{mL}^{-1}$  or higher.

It was clear that after the initial rapid increase during the first 3-5 days post-inoculation (data not shown), the bacterial population in all clones reached a plateau level and subsequently showed no significant increase over time (Figure 10). It should be noted that from 35 samples of different feed microalgae examined on TCBS agar, no presumptive

Vibrionaceae were detected (minimum level of detection =  $\log_{10}$  0.5 units  $\text{mL}^{-1}$ ).

### 2.3 Production of Oyster Larvae.

In this production season only 7 of 13 batches of larvae were successfully reared through to the ready-to-set stage (Table 12). A yield of  $30.5 \times 10^6$  ready-to-set larvae was obtained, representing a survival rate of only 1.3%, with a mean development time of 23.5 days for the first eyed (ready-to-set) larvae. The remainder of larvae was put into set over the next 2-5 days.

Larval batches developed in 3 typical ways, as shown in the growth curves in Figure 11. Figures 11A, 11B, and 11C represent 3, 3 and 7 batches of larvae respectively. Figure 11A shows minimal growth, with major (25-90%; m) losses of larvae at 4-6 days of age and a total (> 90%; t) loss by day 7-10.

Figure 11B shows slow growth of animals until day 10 or 11, associated with small (15-25%; s) losses. The growth rate of the surviving larvae increased until the larvae reached 240-270  $\mu\text{m}$  in size. At this latter stage their growth halted and losses of the animals increased rapidly until the vast majority of the larvae was dead or moribund.

Figure 11C is typical of the batches which reached the ready-to-set (r) stage. The growth rate of these batches was moderately even over time. Nevertheless, a small or major loss was sustained occasionally. The mean age of 23.5 days at which ready-to-set larvae were first seen was considered slow, in developmental terms (M.Fraser, pers.comm.).

Table 11. Characteristic use of feed microalgae (500 L bag cultures) fed to larval oysters during the 1983/84 production season.<sup>a</sup>

Clone <sup>b</sup>	Number of bags used	Time to first use (days <sup>c</sup> )	Volume per use (L <sup>c</sup> )	Number of uses (°)	Time between each use (days <sup>c</sup> )	Microalgal cell density (N x 10 <sup>6</sup> cells mL <sup>-1</sup> °)
T.Iso	55	8.7±8.7	94±19	8.9±5.9	2.1±1.4	2.8±0.8
3C	3	5.7±1.2	53±3	8.0±3.6	1.7±0.6	1.6±0.7
C. cal	17	4.7±1.6	84±20	4.4±3.8	1.8±0.6	0.7±0.2
Dun	9	6.7±2.8	83±28	8.3±4.6	2.8±3.3	0.4±0.1

<sup>a</sup>: The growth medium for feed microalgae was prepared with pasteurised-cooled seawater.

<sup>b</sup>: see Abbreviations, page 4.

<sup>c</sup>: mean ± sd.

Table 12. Production of larval oysters reared in high density conditions during the 1983/84 spawning season<sup>a</sup>.

Total no. eggs spawned (10 <sup>6</sup> x N)	Total no. batches spawned	No. batches reared to r-t-s <sup>b</sup> larval stage	Total no. r-t-s larvae (10 <sup>6</sup> x N)	Mean age of first r-t-s larvae (days)	% survival (eggs to r-t-s larvae)
2330	13	7	30.5	23.5	1.3

<sup>a</sup>: Duration = 7 months.

<sup>b</sup>: ready-to-set

## **B. 1984/85 Production Season.**

### **1.0 Introduction.**

Since high levels of bacteria in the 500 L feed microalgal cultures were considered responsible for causing the high larval mortalities in 1983/84, the system for growing feed microalgae was greatly revised prior to the start of 1984/85: viz. a laminar flow cabinet in which flask-flask microalgae sub-cultures was designed and built by hatchery staff; aseptic techniques for microalgae sub-culturing were taught to hatchery staff; the method for preparing seawater growth medium for feed microalgae was changed from the pasteurising/cooling process used in the 1983/84 production season to a 0.2  $\mu\text{m}$  membrane-filtration system.

### **2.0 Microalgal Growth Medium.**

#### **2.1 Seawater Filtration System.**

In conjunction with the use of 0.2  $\mu\text{m}$  membrane-filtration for treatment of seawater to be used as growth medium for the 500 L microalgal bag cultures, a bacteriological sampling regime was designed to monitor the following:

- a. Total (SWAV) and presumptive Vibrionaceae (TCBS) counts in the filtrate.
- b. Colonisation by bacteria of the internal surfaces of the filtration apparatus during normal daily use of the 0.2  $\mu\text{m}$  membrane-filtered seawater delivery system.



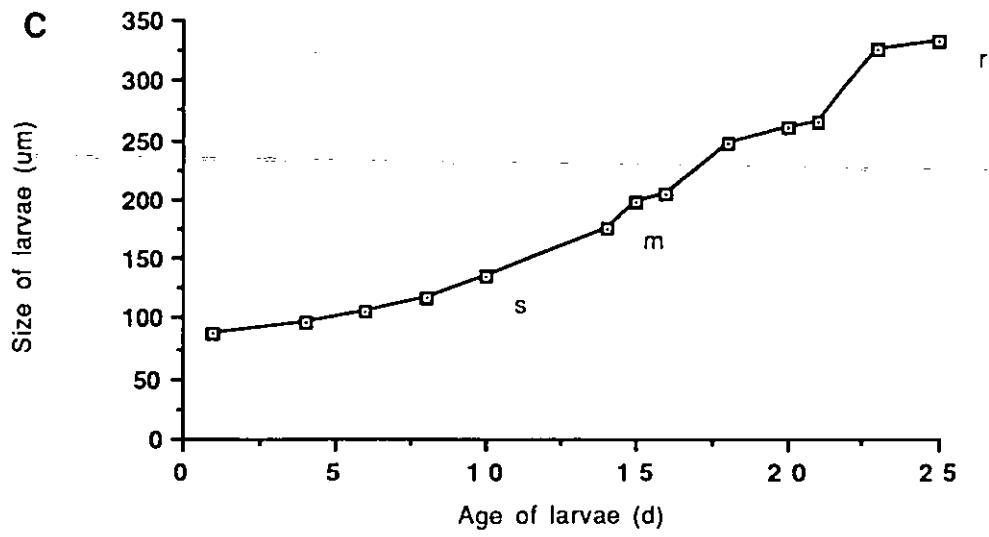
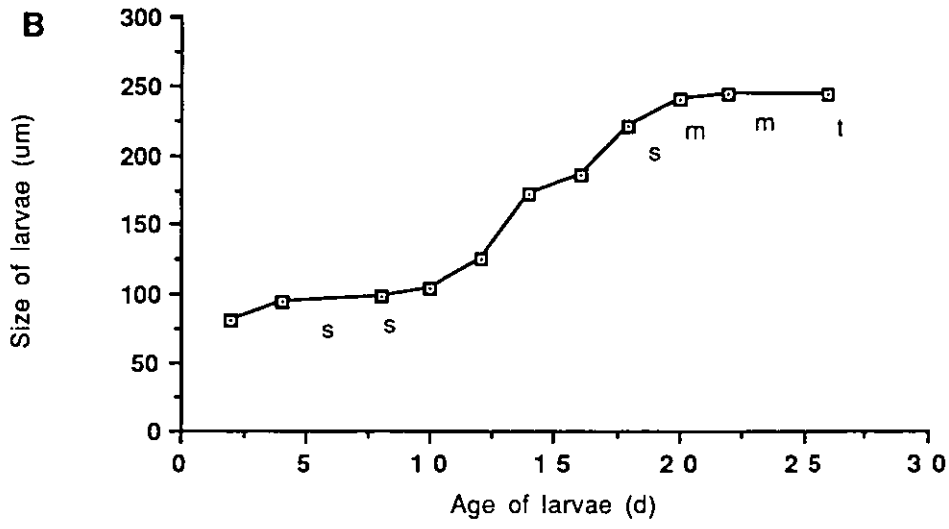
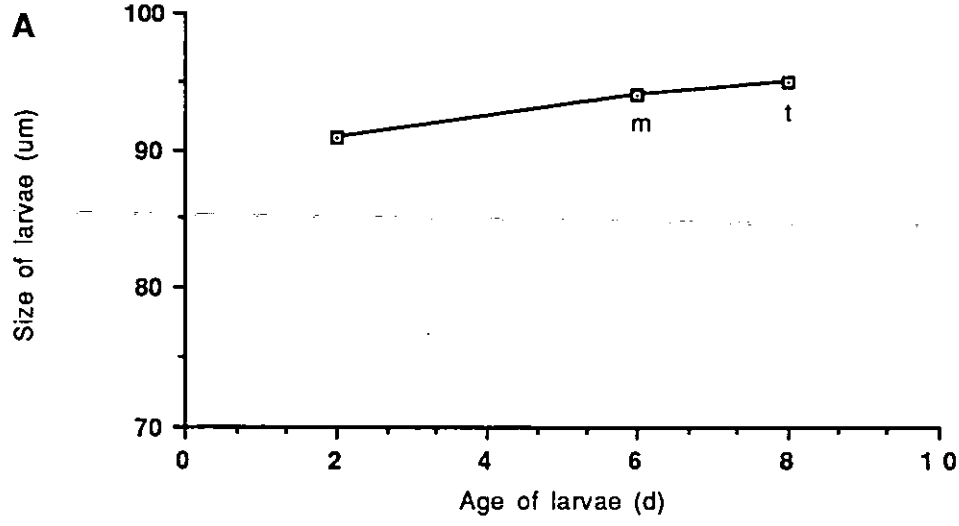
Figure 11. Growth curves for 3 batches of larvae grown during the 1983/84 production season.

s = small (15-25%) loss

m = major (25-90%) loss

t = total (> 90%) loss

r = ready-to-set



c. Development of an effective and practical procedure for the daily sterilisation of the 0.2  $\mu\text{m}$  filter cartridges and all surfaces with which the filtrate came into contact.

A summary of the sequence of the different cleaning methods employed and the results gained during the development of this system are presented in Figure 12 and Table 13. Only viable (SWAV) counts are shown in Table 13. Results for presumptive Vibrionaceae were always low [i.e.  $< 10 \text{ mL}^{-1}$  (spread-plate); 0 or + (swab; Figure 9)] and varied considerably within, and between, treatments. These levels were not considered indicative of the efficacy of the cleaning method used.

The results gained from tests on the original system for 0.2  $\mu\text{m}$  membrane-filtration of seawater used in the hatchery (Table 13, Treatment A) revealed that the initial method for the daily washing and cleaning of the filtration equipment was insufficient to reduce the bacterial biofilm present on the internal surfaces of the 0.2  $\mu\text{m}$  filter housing to below that detectable by the swab technique. This situation was unsatisfactory, as it caused the filtrate to become rapidly contaminated by bacteria sloughing from the housing and delivery line walls before it entered the 500 L culture bags.

The problem was overcome by passing steam through the entire 0.2  $\mu\text{m}$  filter unit (Table 13, Treatment B). The technique achieved complete disinfection of the filter cartridge and housing (determined by the swab technique) which resulted in the delivery of seawater of consistently low bacterial numbers, until the 0.2  $\mu\text{m}$  filter membranes started to break down (Table 13, Treatment C).

The filter-membrane breakdown occurred because the 0.2  $\mu\text{m}$  filter cartridges being used were not designed to withstand repeated autoclaving.

This problem was partially rectified by using one cartridge per day on a 7 or 8 day cycle. Thus the bacterial quality of the seawater delivered by a cartridge could be analysed before that cartridge was used again. If the results indicated a breakdown of the filter membrane the cartridge was discarded.

The monitoring schedule did not, however, allow for cartridges which had broken down after the last bacteriological test. Thus faulty cartridges were detected only after they had been used to deliver seawater to the 500 L microalgal bag cultures. This resulted in the increase in bacterial levels indicated in Table 13, Treatment C.

Modification of the membrane-filtration system ensued with the purchase of higher quality 0.2  $\mu\text{m}$  filter cartridges which, according to the manufacturers specifications, could withstand repeated autoclaving and/or steaming.

This system worked well for a number of months, producing seawater containing very low levels of bacteria, similar to those in Table 13, Treatment B. However, problems were encountered when the acrylic filter housings for the 0.2  $\mu\text{m}$  cartridges started to break down due to the repeated steam treatments.

When this occurred the hatchery manager ordered that all steam disinfection of the filtration system be stopped. From this time onward all filter components were only washed in hot (approx. 60°C) tap water and air dried at room temperature between uses.

Although hatchery staff increased the care used when cleaning the filter components, they could not totally disinfect the system. This led to an increase in the bacterial levels detected in all parts of the filtration system downstream of the 0.2  $\mu\text{m}$  filter cartridge (Table 13, Treatment D). Notwithstanding this, the levels of bacteria entering the 500 L bags were still much lower than those in the pre-filtered seawater.

Table 13. Levels of viable (SWAV) bacteria at sites along the 0.2  $\mu\text{m}$  seawater filtration system.

.....TREATMENT.....

Sample	A		B		C		D		E	
site <sup>a</sup>	AM <sup>b</sup>	PM <sup>c</sup>	AM	PM	AM	PM	AM	PM	AM	PM <sup>f</sup>
1 <sup>d</sup>	2.8	nd <sup>g</sup>	3.0	nd	3.0	nd	2.9	nd	2.9	nd
2 <sup>d</sup>	3.4	nd	3.7	nd	3.1	nd	3.2	nd	3.3	nd
3 <sup>d</sup>	4.2	nd	4.0	nd	4.2	nd	4.1	nd	4.0	nd
4 <sup>d</sup>	3.0	3.2	3.2	3.3	3.2	3.3	3.1	3.4	3.2	3.4
5 <sup>e</sup>	+	+++	ng <sup>h</sup>	ng	ng	++	+	+++	ng	ng
6 <sup>e</sup>	+	+++	ng	++	ng	++	+	+++	ng	++
7 <sup>e</sup>	+	+++	ng	ng	ng	+++	ng	+++	ng	ng
8 <sup>f</sup>	100	100	0.3	0.5	100	500	10	150	0.01	0.1
9 <sup>e</sup>	ng	++	ng	ng	ng	++	+	+++	ng	ng
10 <sup>f</sup>	100	300	0.2	0.7	50	300	10	1000	0.1	0.2
11 <sup>e</sup>	ng	ng	ng	ng	ng	ng	ng	ng	ng	ng
12 <sup>d</sup>	ng	ng	ng	ng	ng	ng	ng	ng	ng	ng

<sup>a</sup>: see Figure 12.

<sup>b</sup>: Sample taken in the morning immediately prior to system startup.

<sup>c</sup>: Sample taken in the afternoon immediately prior to system shutdown.

<sup>d</sup>:  $\text{Log}_{10}$  bacteria  $\text{mL}^{-1}$ .

<sup>e</sup>: See Figure 9.

<sup>f</sup>: Bacteria  $\text{mL}^{-1}$ .

<sup>g</sup>: not determined.

<sup>h</sup>: no growth.

Key for Table 13.<sup>a</sup>

Sample number	Sample	Sampling technique <sup>b</sup>
1	Intake seawater.	Spread plate
2	Settling tank water.	Spread plate
3	1 µm cartridge-filtered water <del>a</del>	Spread plate
4	0.45 µm pad-filtered water.	Spread plate
5	Seawater transfer hose.	Swab
6	External surface of 0.2 µm filter cartridge.	Swab
7	Internal surface of 0.2 µm filter cartridge.	Swab
8	0.2 µm membrane-filtered seawater direct from filter housing outlet.	Membrane
9	Internal surface of MTLA.	Swab
10	0.2 µm membrane-filtered seawater from free end of MTLA.	Membrane
11	Nutrient solution delivery line.	Swab
12	Nutrient solution.	Spread plate

Sterilisation regimes<sup>c</sup>.

- A. Pre-filter housings and cartridges and 0.2 µm housing washed in hot (approx. 60°C) tap water and air dried at room temperature. MTLA, nutrients and 0.2 µm membrane-filter cartridge autoclaved.
- B. As for A but complete filtration unit assembled and steamed before use.
- C. As for C but with 0.2 µm filters breaking down due to steaming.
- D. All filtration components washed in hot (approx. 60°C) tap water and air dried at room temperature.
- E. Stainless steel 0.2 µm filter housing, cartridge and delivery hose autoclaved as a single unit prior to use each day. 0.2 µm membrane integrity tested *in situ* prior to use (1985/86 production season only).

<sup>a</sup>: See Figure 12 for positions of sample sites.

<sup>b</sup>: see Materials and Methods section B.1.1.1.

<sup>c</sup>: See Materials and Methods section A.1.2.2 for a complete description of sterilisation techniques.

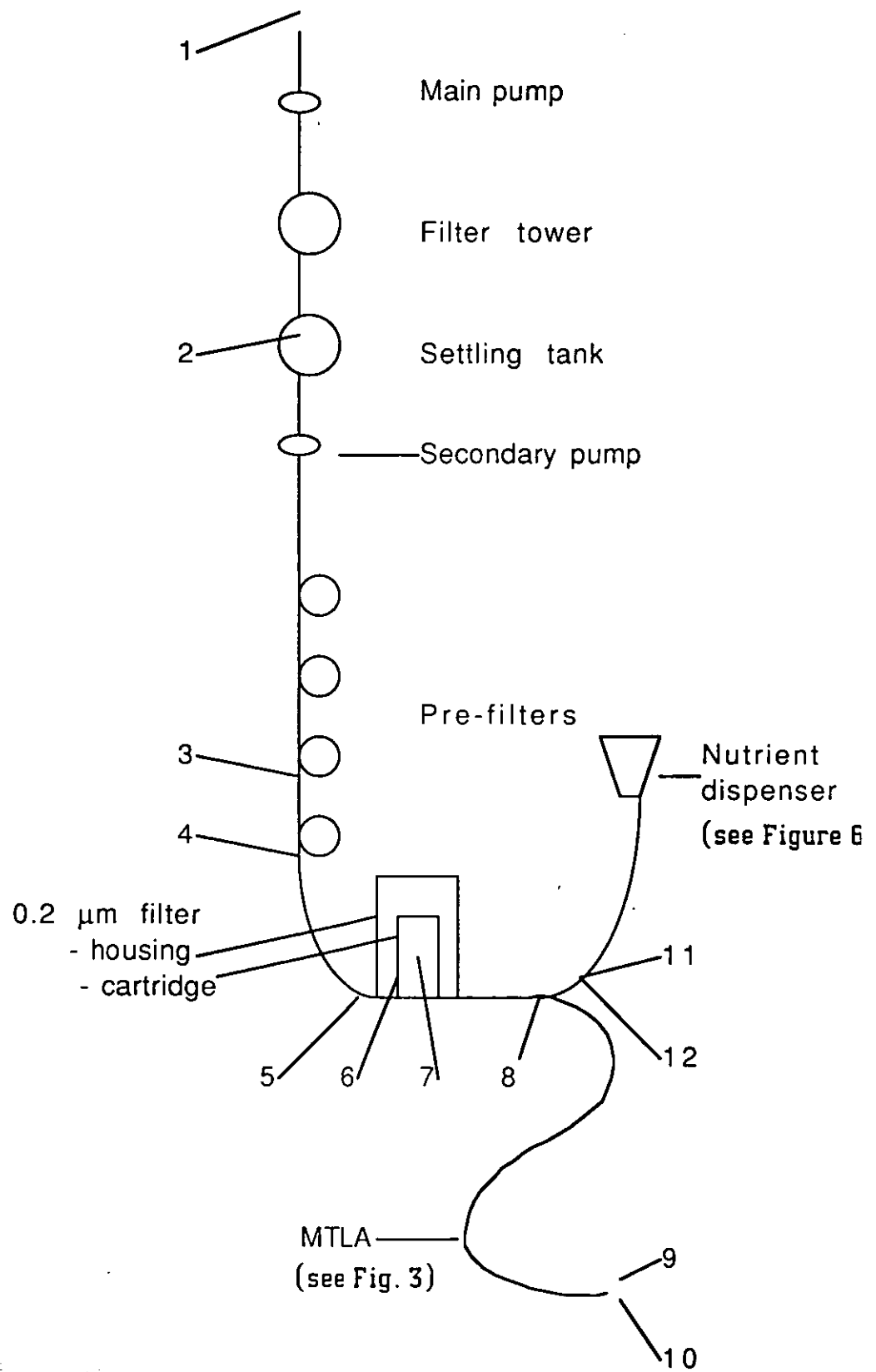
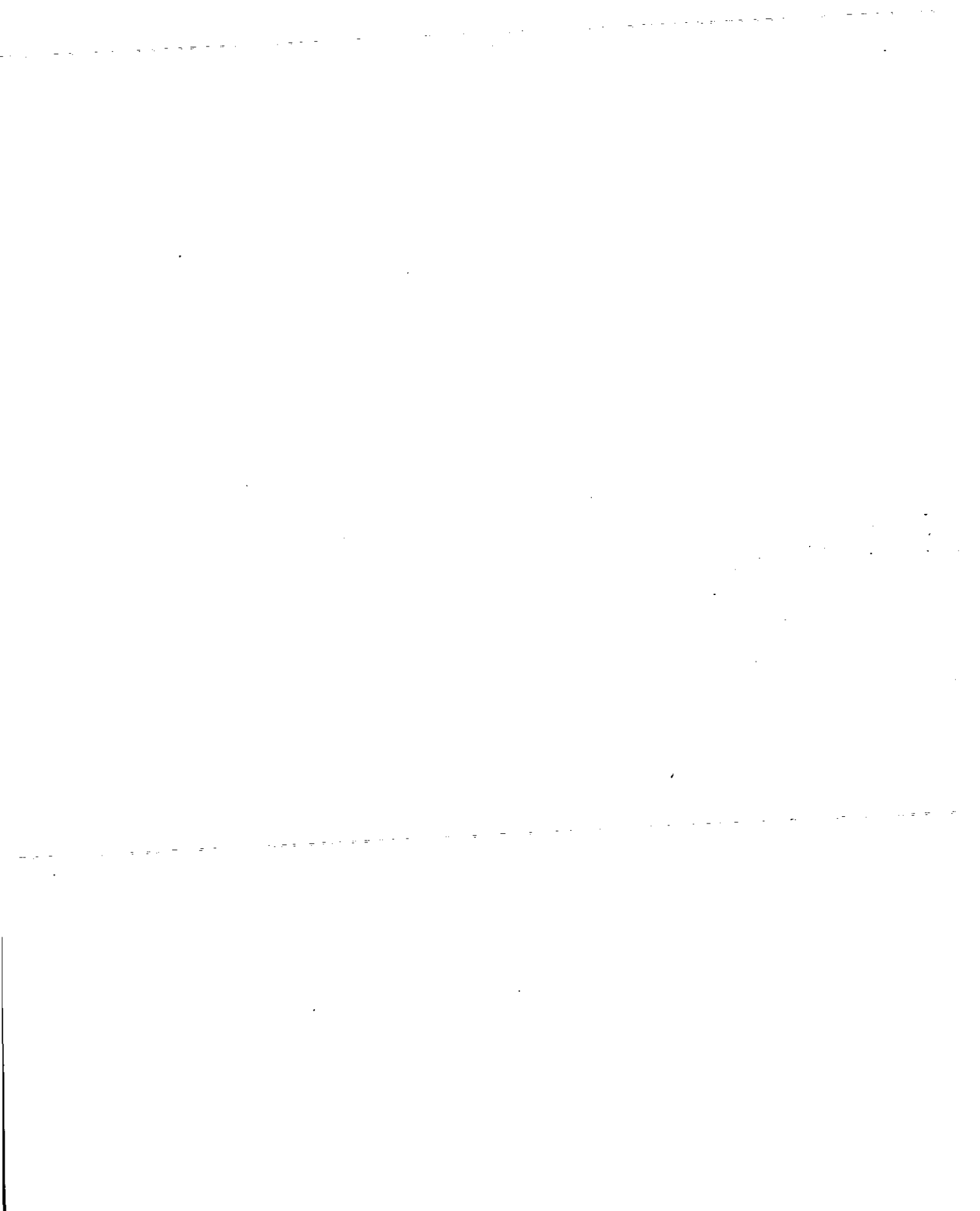


Figure 12. Diagram of sampling sites for bacteriological monitoring of seawater filtration system (see Table 13 for details).





This system of cleaning the filtration system was used until the end of the 1984/85 production season.

## 2.2 0.2 $\mu\text{m}$ Membrane-Filtered Seawater.

Initial counts of viable (SWAV) bacteria in the 0.2  $\mu\text{m}$  filtrate were obtained in duplicate using membrane discs of 0.2 and 0.1  $\mu\text{m}$  pore size. There was no significant difference (Students t-test) between the mean  $\pm$  sd counts for each disc type (Appendix 4). It was therefore decided to use discs of 0.2  $\mu\text{m}$  pore size for the monitoring program as these were faster to use and were also considerably cheaper.

In 1984/85 the mean count of viable (SWAV) bacteria in 0.2  $\mu\text{m}$  membrane-filtered seawater was very low. Levels varied between  $\log_{10}$  0 and 2.3 units  $100\text{mL}^{-1}$  with a mean  $\pm$  sd of  $\log_{10}$   $1.5 \pm 1.7$  units  $100\text{mL}^{-1}$  (Table 14). This was significantly lower than the level in pasteurised-cooled seawater growth medium used in 1983/84, which was  $\log_{10}$   $4.1 \pm 0.8$  units  $\text{mL}^{-1}$ . (Table 10) (Students t-test,  $P < 0.001$ ).

The highest levels were recorded during the periods of 0.2  $\mu\text{m}$  filter membrane breakdown and/or no steam disinfection of the 0.2  $\mu\text{m}$  filter housing (Table 13, Treatments A, C and D).

## 3.0 Microalgal Culture.

### 3.1 Laminar Flow Unit.

Bacteriological testing of the conditions provided in the laminar flow cabinet, while microalgal sub-culturing was being performed, failed to detect any airborne microorganisms. Controls plates exposed to the air outside the cabinet consistently grew bacterial and fungal colonies after incubation.

Table 14. Counts of viable bacteria (SWAV; log<sub>10</sub> units)

Production season	Bacteria <sup>a</sup> in microalgal culture medium <sup>b</sup> (N)	5 L starter culture of microalgae <sup>c</sup> (N)	Bacteria <sup>a</sup> in 500 L bag cultures	% of 500 L bag cultures with > 6.3 log <sub>10</sub> bacteria mL <sup>-1</sup>
1984/85	1.5±1.7 100 mL <sup>-1</sup> (240)	axenic (238)	5.8±0.6 mL <sup>-1</sup>	20.6
1985/86	1.5±1.5 100 mL <sup>-1</sup> (118)	axenic, non-axenic (427)	5.9±0.4 mL <sup>-1</sup>	10.9

<sup>a</sup>: mean ± sd.

<sup>b</sup>: 0.2 µm membrane-filtered seawater.

<sup>c</sup>: used as inoculum for 500 L microalgal cultures.

### 3.2 Microalgal Flask Cultures.

All microalgal flask cultures were free from viable (SWAV) bacteria during the 1984/85 production season.

### 3.3 Harvest Regime and Bacterial Content of Feed Microalgae.

#### 3.3.1 Harvest regime.

The microalgal cultures grown in 500 L bags using 0.2  $\mu\text{m}$  membrane-filtered seawater growth medium were harvested on a semi-continuous basis at suitable cell densities while in the log phase. The characteristic use of feed microalgae bags in 1984/85 is shown in Table 15. In total, 200 bags were used, compared to 84 bags in the previous season when far fewer animals were produced. It was notable that microalgae cultured in 0.2  $\mu\text{m}$  membrane-filtered seawater had very similar harvest characteristics (e.g. the time to first use of the bags of feed microalgae, the volume of microalgae used at each harvest, the number of uses of each bag and the time between each harvest) to that cultured in pasteurised-cooled seawater (Table 15). A further advantage associated with the use of membrane-filtered seawater was that microalgal culture bags filled with this growth medium could be inoculated immediately with a starter culture of microalgae and sterile growth nutrients. In contrast, up to 36 hours was needed for pasteurised seawater to cool in the 500 L bags before they could be inoculated. A similar time saving was made with regard to bags refilled (after harvest) with membrane-filtered seawater.

Larvae were fed a mixed diet of microalgal cultures as shown in Table 9.

Table 15. Characteristic use of feed microalgae (500 L bag cultures) fed to larval oysters during the 1984/85 and 1985/86 production seasons.<sup>a</sup> (c.f. Table 11.)

Clone <sup>b</sup>	Production season	Number of bags used	Time to first use (days <sup>c</sup> )	Volume per use (L <sup>c</sup> )	Number of uses (°)	Time between each use (days <sup>c</sup> )	Microalgae cell density (N x 10 <sup>6</sup> mL <sup>-1</sup> °)
T.Iso	1984/85	63	5.6±2.1	112±22	8.4±5.6	1.6±0.7	ND <sup>c</sup>
	1985/86	20	6.5±3.8	107±29	14.8±9.3	1.9±1.2	3.1±0.8
3C	1984/85	20	5.2±1.3	118±40	6.8±5.2	1.2±0.3	ND
	1985/86	6	5.8±3.1	62±10	6.3±3.5	1.1±0.2	1.1±0.6
3H	1984/85	40	5.1±1.4	126±35	6.1±4.5	1.4±0.6	ND
	1985/86	28	4.6±1.4	107±34	5.4±3.2	1.2±0.6	3.8±1.0
C. cal	1984/85	43	4.3±1.1	112±37	4.1±3.0	1.3±0.4	ND
	1985/86	7	3.6±0.8	81±29	2.7±1.4	1.3±1.2	5.6±2.0
Dun	1984/85	24	6.7±3.2	110±36	6.4±3.5	2.4±3.5	ND
	1985/86	7	7.6±2.2	60±24	4.3±5.0	1.3±1.5	1.1±0.4
Pav	1985/86	10	5.7±1.9	109±12	14.4±6.1	1.8±0.7	2.7±0.9
Tet	1985/86	4	5.3±0.9	74±11	8.2±7.1	1.2±0.2	1.0±0.7

<sup>a</sup>: The growth medium for feed microalgae consisted of 0.2 µm membrane-filtered seawater.

<sup>b</sup>: See Abbreviations, page 4; <sup>c</sup>: mean ± sd.; <sup>d</sup>: Not Determined.

### 3.3.2 Bacteriological Results.

The levels of viable (SWAV) bacteria in 500 L microalgal cultures grown in 1984/85 are shown in Figures 13 and 14. After a rapid rise in the first (pre-harvest) 1-6 days (Figure 13) the bacterial numbers tended to plateau and did not increase over time except for *C. calcitrans* which continued to multiply (Figure 14). The mean numbers of viable (SWAV) bacteria in cultures of all clones fed to oyster larvae were lower than in the previous season (Table 16). The degree of significance (Students t-test) of the decreases in mean bacterial numbers for each clone were as follows; *Isochrysis* sp. and *C. calcitrans*  $P < 0.001$ ; *D. tertiolecta*  $P < 0.01$ ; *Ch. salina*  $P < 0.1$  (no significant difference).

The overall mean bacterial count in microalgae fed to the oyster larvae was  $\log_{10} 5.8 \pm 0.6$  units  $\text{mL}^{-1}$  (Table 14) and was significantly lower than the count of  $\log_{10} 6.4 \pm 0.8$  units  $\text{mL}^{-1}$  in 1983/84 (Table 10) (Student's t-test,  $P < 0.001$ ). This represented a four-fold decrease in the bacterial load encountered by the larvae.

Presumptive *Vibrio* spp. (TCBS) were detected in only 3 of 93 samples of feed microalgae. The *Vibrio* count varied from  $\log_{10} 2.4$ - $2.9$  units  $\text{mL}^{-1}$ .

Two major sources of the bacteria which grew in the 500 L microalgal cultures were identified. One source was the  $0.2 \mu\text{m}$  membrane-filtered seawater, which rarely had counts below  $1 \text{ } 100 \text{ mL}^{-1}$ . The second opportunity for contamination occurred during the setting up of the 500 L bags. The various fittings were installed using aseptic techniques (see Materials and Methods section A.3.2.2).

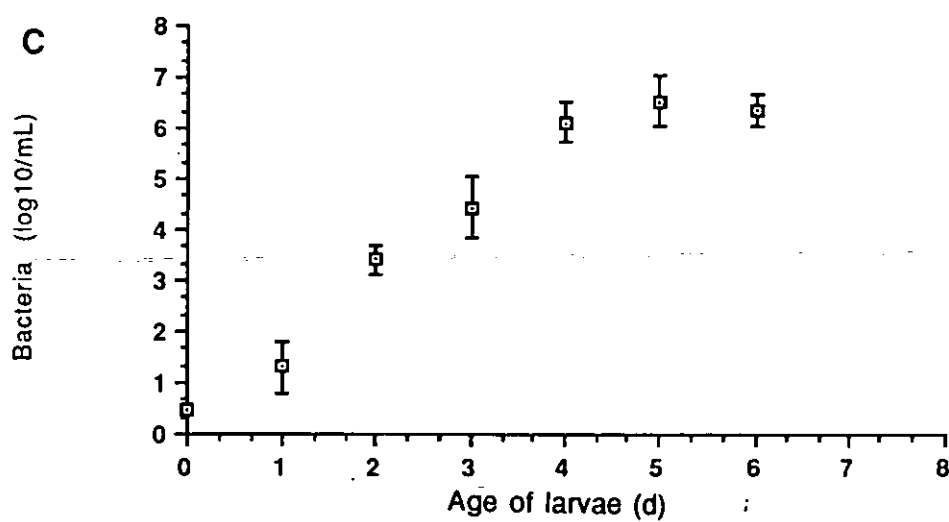
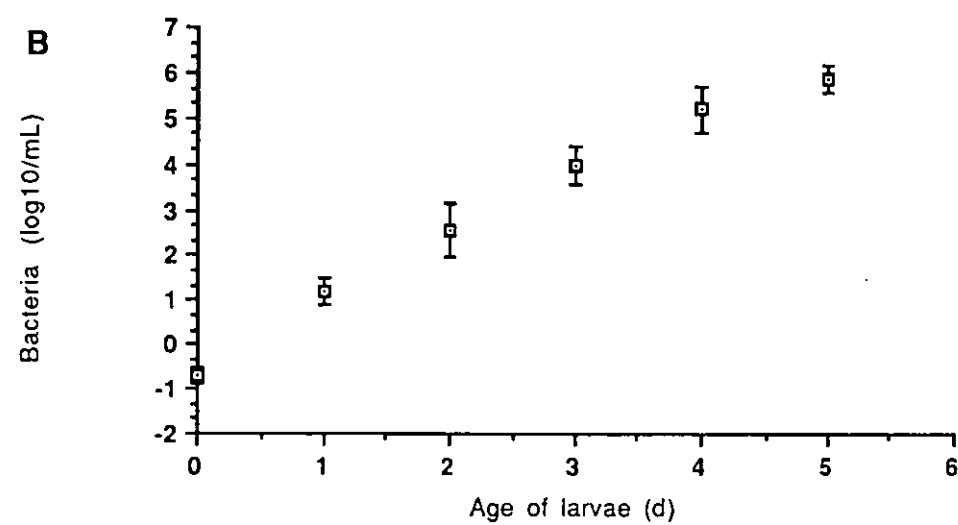
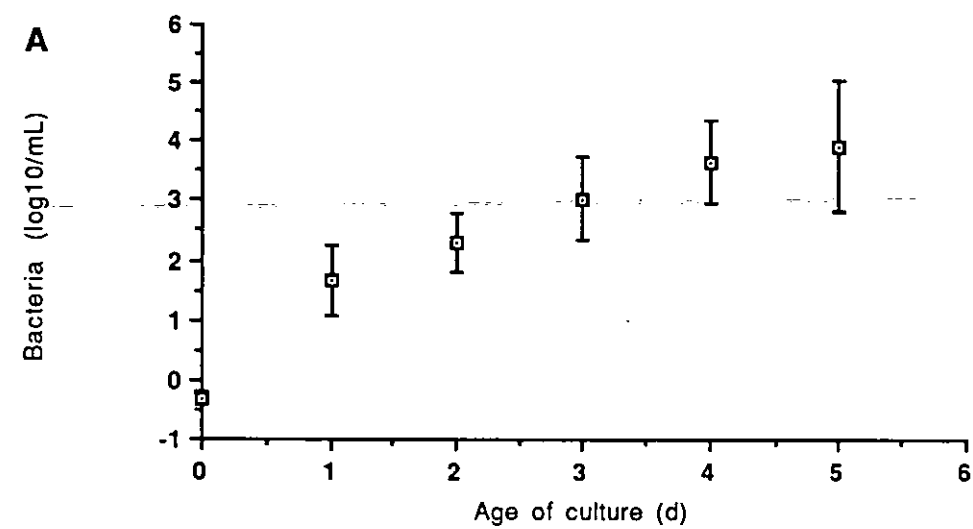
Figure 13. Viable (SWAV) bacteria in pre-harvest 500 L  
microalgal cultures in 1984/85.

Points with bar markers represent mean  $\pm$  sd value  
on each day of sampling.

Single points indicate counts for which  
only one value was recorded.

Key.

- A. *Isochrysis* sp. (T.Iso)
- B. *Chroomonas salina*
- C. *Thalassiosira pseudonana*
- D. *Chaetoceros calcitrans*
- E. *Dunaliella tertiolecta*



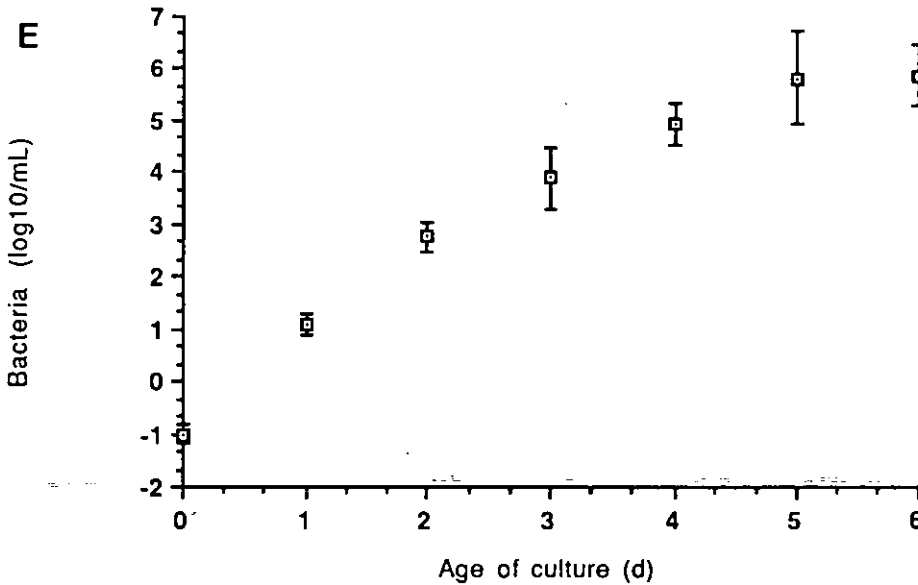
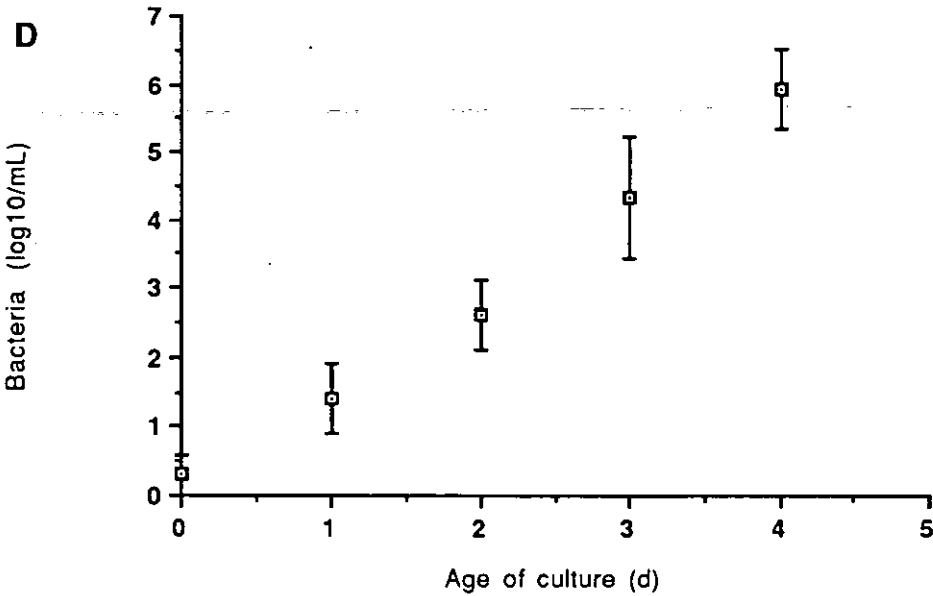
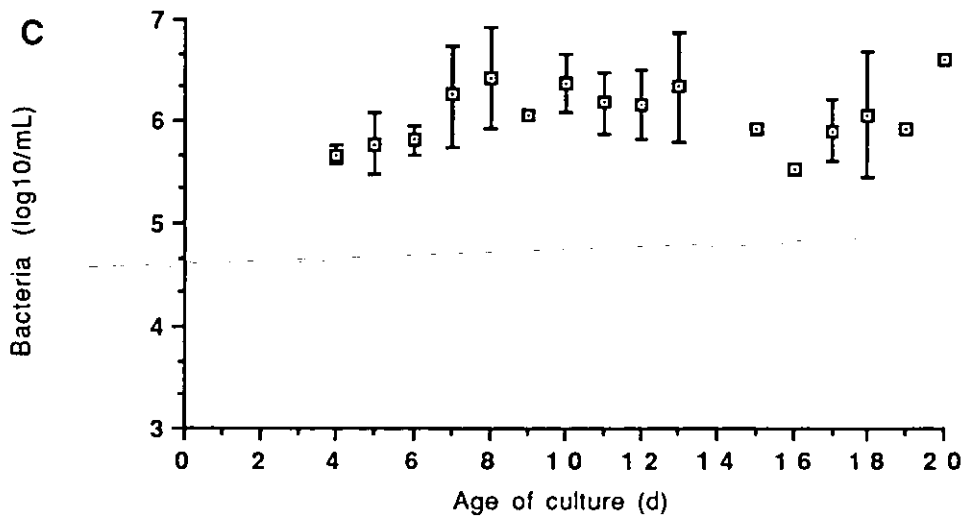
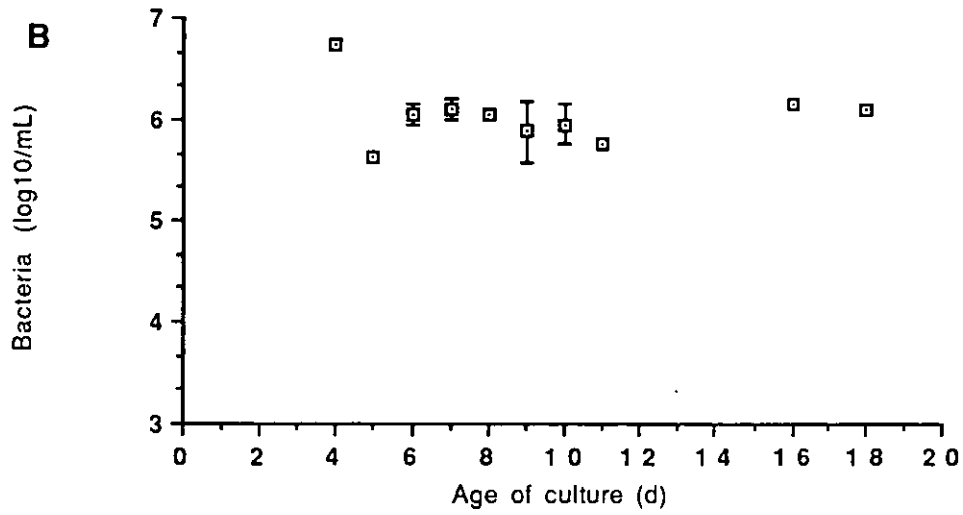
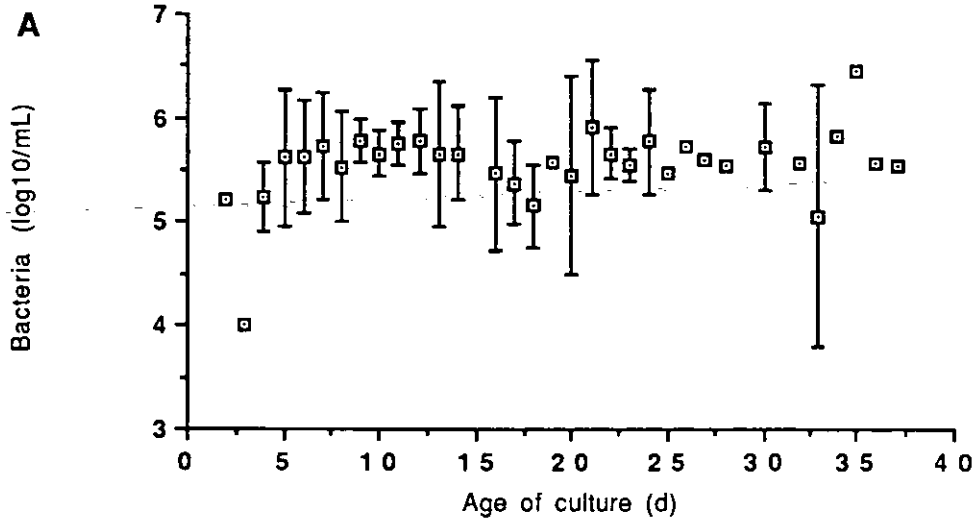




Figure 14. Viable (SWAV) bacteria in 500 L semi-continuously harvested microalgae cultures fed to larvae in 1984/85. Points with bar markers represent mean  $\pm$  sd value on each day of sampling. Single points indicate counts for which only one value was recorded.

Key.

- A. *Isochrysis* sp. (T. Iso)
- B. *Chroomonas salina*
- C. *Thalassiosira pseudonana*
- D. *Chaetoceros calcitrans*
- E. *Dunaliella tertiolecta*



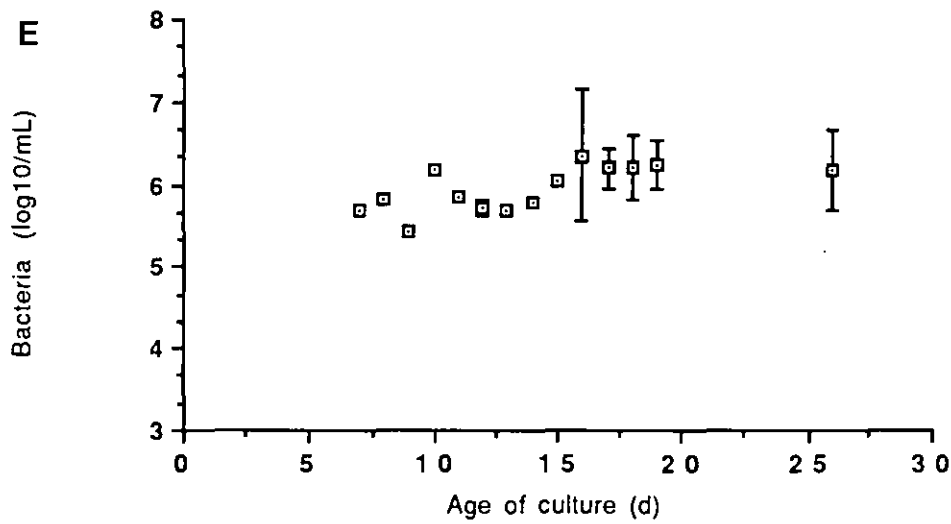
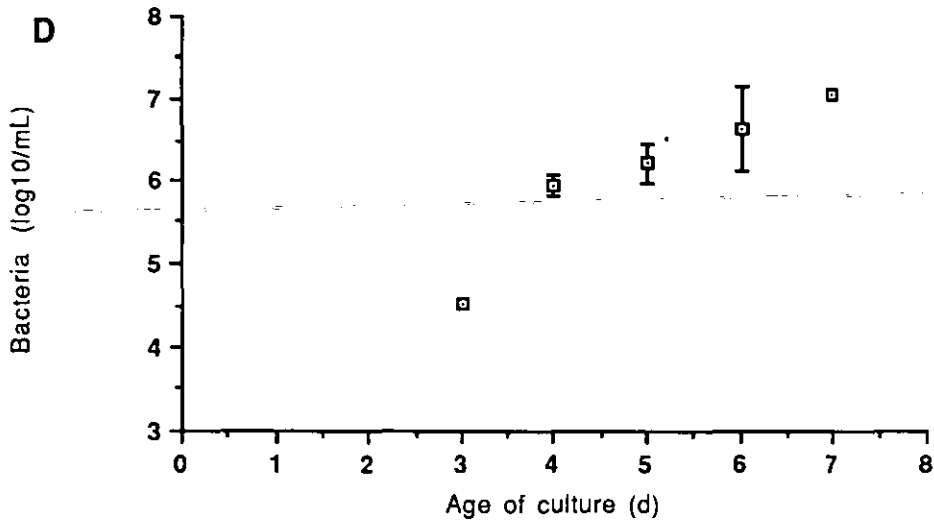


Table 16. Viable (SWAV) bacteria in microalgal cultures fed to oyster larvae.  
[mean  $\pm$  sd log<sub>10</sub> units mL<sup>-1</sup> (N)]

Production season	..... Clone of Microalgae <sup>a</sup> .....						
	T. Iso	3C	3H	C. cal.	Dun.	Pav.	Tet.
1983/84 <sup>b</sup>	6.3 $\pm$ 0.7 (119)	6.3 $\pm$ 1.1 (7)	NU <sup>c</sup>	7.2 $\pm$ 0.4 (16)	6.5 $\pm$ 0.8 (22)	NU	NU
1984/85	5.6 $\pm$ 0.6 (138)	6.0 $\pm$ 0.3 (15)	6.1 $\pm$ 0.4 (52)	6.2 $\pm$ 0.7 (13)	6.1 $\pm$ 0.3 (20)	NU	NU
1985/86	5.8 $\pm$ 0.3 (180)	6.2 $\pm$ 0.4 (19)	5.9 $\pm$ 0.3 (94)	5.7 $\pm$ 0.3 (8)	5.6 $\pm$ 0.3 (19)	5.9 $\pm$ 0.3 (87)	5.9 $\pm$ 0.3 (20)

<sup>a</sup>: see Abbreviations, page 4.

<sup>b</sup>: Values from 1983/84 (Table 10) are repeated here to facilitate comparison with values from subsequent seasons.

<sup>c</sup>: Not Used.

### 3.4 SEM Examination of Microalgal Cells.

Scanning electron microscopic examination of representative samples<sup>a</sup> of microalgal cells from 500 L cultures revealed that only 6 of 20 (30%) samples examined contained cells colonised by bacteria. All clones except *P. lutheri* were amongst those colonised by bacteria. Figures 15A and 15B show typical examples of microalgal cells colonised by bacteria.

### 4.0 Production of Oyster Larvae.

The production figures for 1984/85 are shown in Table 17. These demonstrate a marked improvement compared to 1983/84, when only  $30.5 \times 10^6$  ready-to-set larvae were produced. The increase was mainly due to a rise in the larval survival rate, from 1.3% to 3.2%. Only 1 of 12 batches was lost from bacterial disease in 1984/85 compared to 6 of 13 batches lost in 1983/84. The growth curves of the 11 successfully reared batches were similar. The mean growth curve of these batches (Figure 16) shows an even rate of development. Even so, in several batches (to be discussed later), small and occasionally major losses of larvae due to bacterial infection were experienced.

The mean time at which ready-to-set larvae were first seen was 18.6 days (Table 17), 5 days earlier than in 1983/84 (Table 12).

## C. 1985/86 Production Season.

### 1.0 Introduction.

Because of the important role of 0.2  $\mu\text{m}$  membrane-filtered seawater in the improved production of the 1984/85 season, it was again used for the culture of feed microalgae in the following season.

<sup>a</sup>. Microalgal samples were obtained from 4 different cultures of each clone used during the 1984/85 production season. Each sample was collected during the first week that the selected culture was being fed to larvae.

Figure 15. Bacteria associated with the surface of  
microalgal cells cultured in 500 L bags.

A. *Chroomonas salina*

SEM x 11,500; bar = 1  $\mu\text{m}$ .

B. *Dunaliella tertiolecta*

SEM x 7800, bar = 1  $\mu\text{m}$ .

A

  
 $1\mu$ 

B


  
 $1\mu$

Table 17. Production of larval oysters reared in high density conditions during successive spawning seasons.

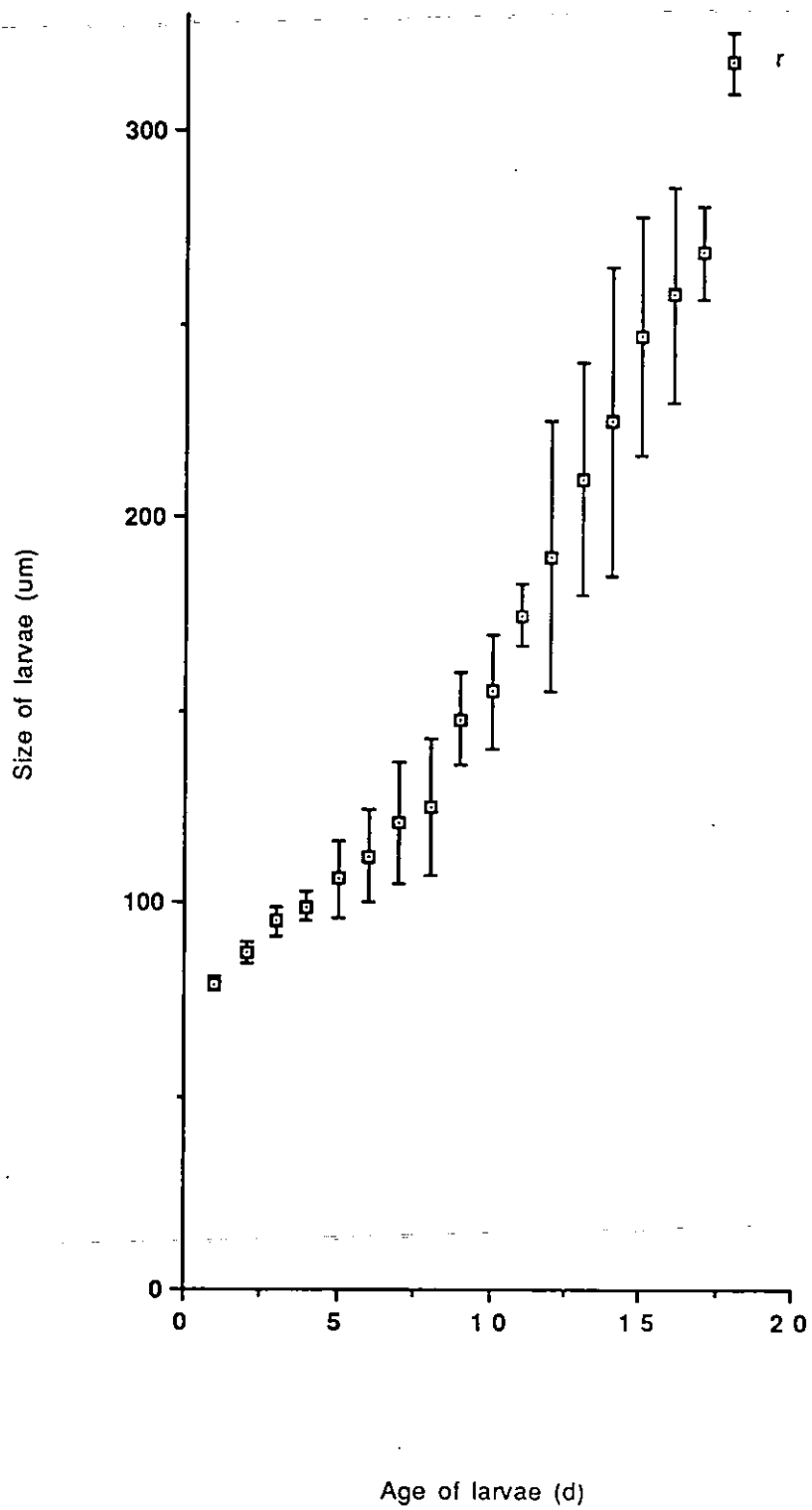
Spawning season (duration)	Total no. eggs spawned ( $10^6 \times N$ )	Total no. batches spawned	No. batches reared to r-t-s <sup>a</sup> larval stage	Total no. r-t-s larvae ( $10^6 \times N$ )	Mean age of first r-t-s larvae (days)	% survival (eggs to r-t-s larvae)
1984/85 (7 months)	3380	12	11	106.5	18.6	3.2
1985/86 (6 months)	2030	9	8	94.2	18.5	4.6

<sup>a</sup>: ready-to-set



Figure 16. Growth curve (mean  $\pm$  sd) of larvae in the 8 healthy batches reared in 1984/85.

r = ready-to-set



## 2.0 Microalgal Growth Medium.

### 2.1 Seawater Filtration System.

modified  
Bacteriological tests for levels of contamination of the various parts of the seawater membrane-filtration system (Table 13, Treatment E) show that the (modified) 0.2  $\mu\text{m}$  membrane-filtration system (see Materials and Methods section A.1.2.2.3) was very effective at producing large volumes of seawater with very low bacterial counts. The possibility of contamination of sterile (autoclaved) filter components during assembly was eliminated, the possibility of using a faulty 0.2  $\mu\text{m}$  membrane-filter cartridge was greatly reduced. This new system invariably produced seawater of acceptable bacterial quality.

### 2.2 0.2 $\mu\text{m}$ Membrane-Filtered Seawater.

The mean number of bacteria present in the 0.2  $\mu\text{m}$  membrane-filtered seawater in the 1985/86 season was very similar to that of the preceding production season (Table 14). The 1985/86 counts ( $\log_{10}$   $1.5 \pm 1.5$  units  $100\text{mL}^{-1}$ ) showed less variation than the counts from the 1984/85 season ( $\log_{10}$   $1.5 \pm 1.7$  units  $100\text{mL}^{-1}$ ) although their total ranges were similar. This can be attributed to the introduction in the 1985/86 season of aseptic *in situ* integrity testing of the 0.2  $\mu\text{m}$  filter membranes.

## 3.0 Microalgal Culture.

### 3.1 Laminar-Flow Unit.

Results from bacteriological tests of the efficacy of the laminar-flow unit again failed to detect any airborne microorganisms in the working area of the operational unit.

### 3.2 Microalgal Flask Cultures.

Some 5 L flask microalgal cultures were found to be contaminated with viable (SWAV) bacteria during this season (Table 14). This was due to poor aseptic sub-culture techniques employed by a new hatchery staff member, who was subsequently taught the appropriate techniques.

### 3.3 Harvest Regime and Bacterial Content of Feed Microalgae.

#### 3.3.1 Harvest Regime.

Characteristic harvesting of feed microalgae in 1985/86 was very similar to that in the 1984/85 season (Table 15). The smaller volumes of *D. tertiolecta* and *Ch. salina* used in 1985/86 were supplemented with volumes of *Tetraselmis suecica* and *Pavlova lutheri*. These latter two flagellate microalgae were first used in the hatchery in the 1985/86 season. *T. suecica* was fed to larvae from day 10 through to the ready-to-set stage, while *P. lutheri* was fed throughout the larval cycle. The number of harvests of each bag of *Isochrysis* sp. was considerably increased compared to the previous season. It was noted that the use of 0.2  $\mu$ m membrane-filtered seawater in place of pasteurised-cooled seawater as the growth medium resulted in feed microalgae of similar (*Isochrysis* sp., *Ch. salina* 3C) or higher (*C. calcitrans*, *D. tertiolecta*) cell density at harvest (Table 15 cf Table 11).

Larvae were fed a mixed diet of these microalgal cultures as shown in Table 9.

### 3.3.2 Bacteriological Results.

The replication rates of populations of bacteria in the 500 L microalgal cultures in 1985/86 were very similar to those in 1984/85 (Figures 17 and 18).

As in 1984/85, bacterial populations rose rapidly in the first (pre-harvest) 1 - 6 days (Figure 17) and attained a stable level over time, except for *C. calcitrans* which continued to multiply (Figure 18).

Mean counts of viable (SWAV) bacteria in cultures of microalgae fed to larvae are shown in Table 16. Mean levels of bacteria in the microalgal cultures at harvest during this season were significantly lower (Students t-test) than during the preceding season for *Isochrysis* sp. and *D. tertiolecta* ( $P < 0.001$ ), and *T. pseudonana* ( $P < 0.01$ ), but not for *C. calcitrans* and *Ch. salina* ( $P > 0.05$ ).

The overall mean count of bacteria in microalgae fed to the oyster larvae was  $\log_{10} 5.9 \pm 0.4$  units  $\text{mL}^{-1}$  (Table 14), which was similar to the corresponding value for 1984/85 ( $\log_{10} 5.8 \pm 0.6$  units  $\text{mL}^{-1}$ ). It was notable that the size of the standard deviation of the mean count was lower in 1985/86 than in the earlier season. This can be attributed the production of growth medium with a more stable bacterial content. This increase of stability was due to the implementation of an aseptic in-line integrity testing regime which allowed faulty filters to be detected and discarded before use.

Presumptive *Vibrio* spp. (TCBS) were detected in only 3 of 424 (0.7%) samples of feed microalgae. The *Vibrio* count varied from  $\log_{10} 2.08$ - $2.95$  units  $\text{mL}^{-1}$ .

There was no significant difference (Students t-test) between plateau levels of bacteria in 500 L microalgal cultures inoculated with axenic or non-axenic starter cultures (Table 18). Non-axenic starter cultures had

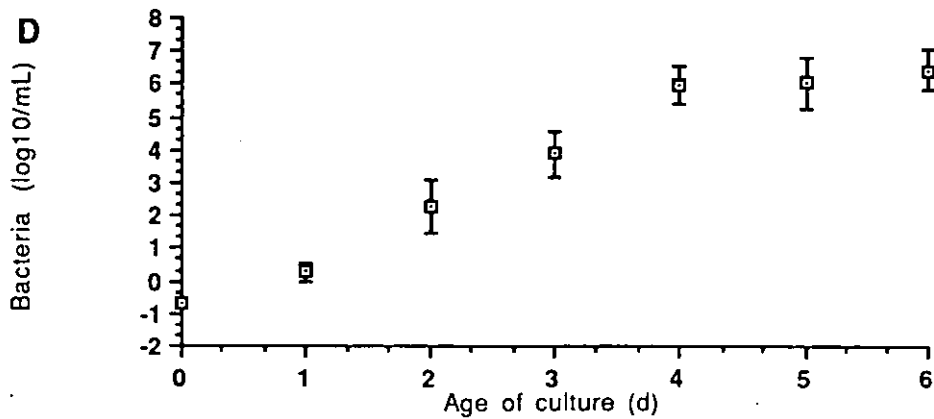
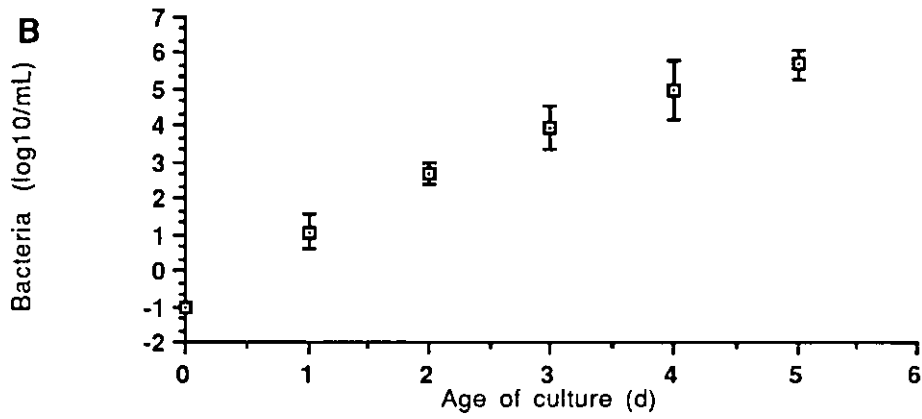
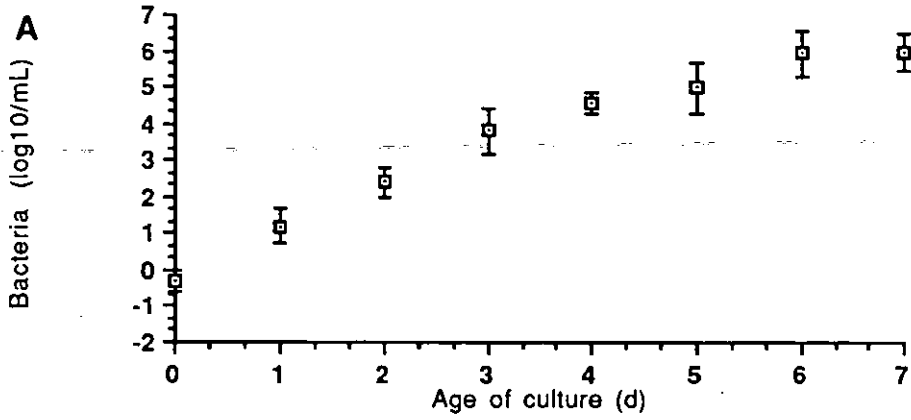
Figure 17. Viable (SWAV) bacteria in pre-harvest 500 L  
microalgal cultures in 1985/86.

Points with bar markers represent mean  $\pm$  sd value  
on each day of sampling.

Single points indicate counts for which  
only one value was recorded.

Key.

- A. *Isochrysis* sp. (T. Iso)
- B. *Chroomonas salina*
- C. *Thalassiosira pseudonana*
- D. *Chaetoceros calcitrans*
- E. *Dunaliella tertiolecta*
- F. *Pavlova lutheri*
- G. *Tetraselmis suecica*



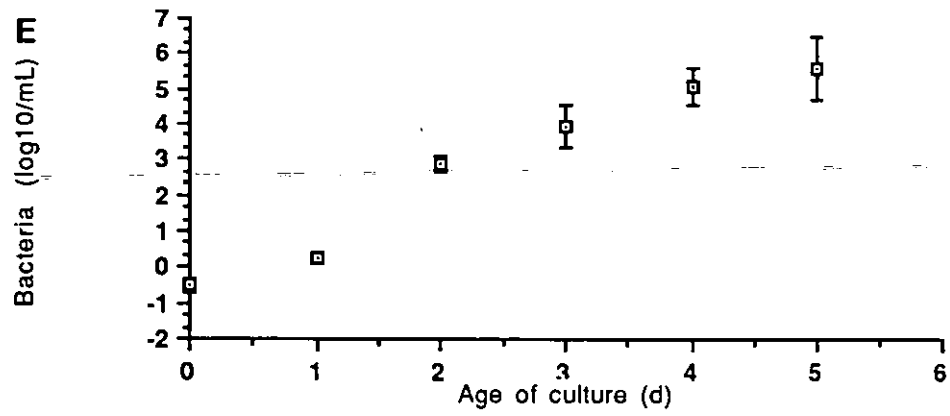
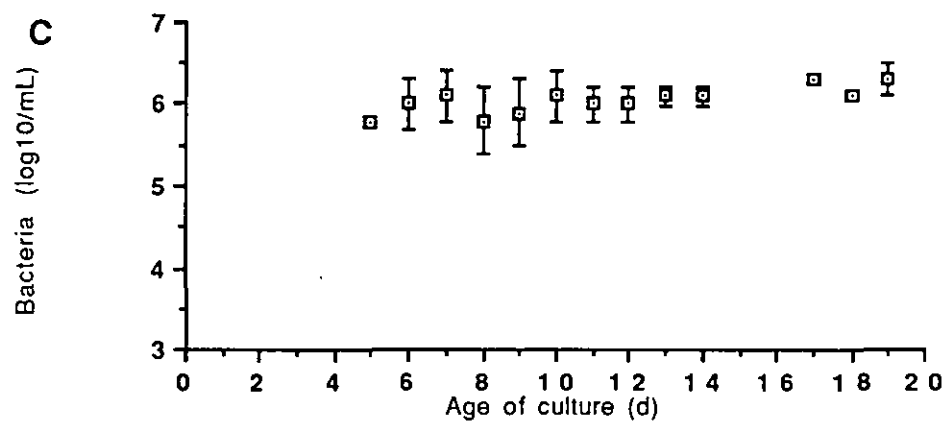
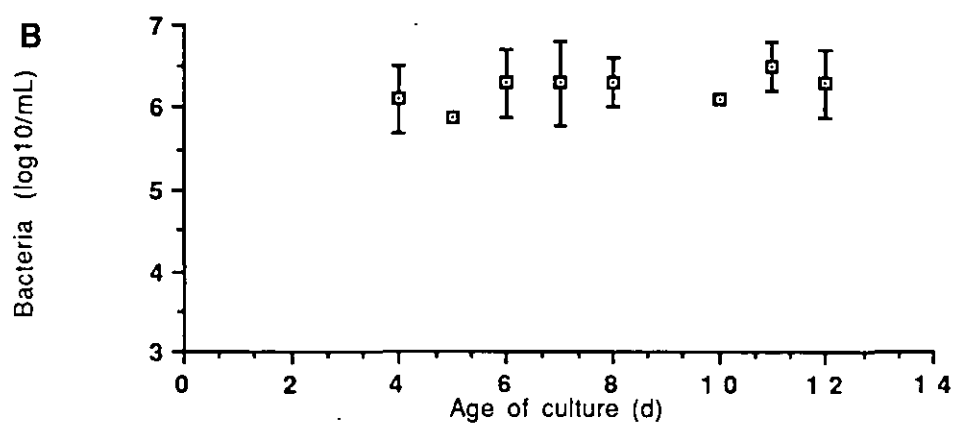
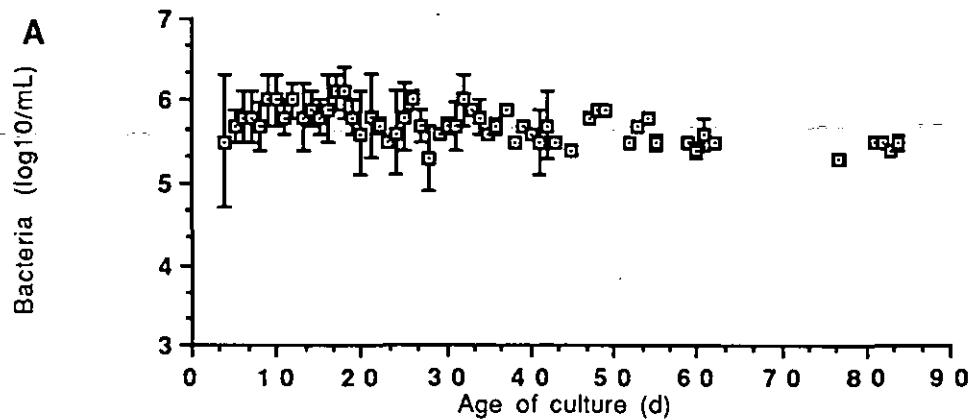




Figure 18. Viable (SWAV) bacteria in 500 L semi-continuously harvested microalgal cultures fed to larvae in 1985/86. Points with bar markers represent mean  $\pm$  sd value on each day of sampling. Single points indicate counts for which only one value was recorded.

Key.

- A. *Isochrysis* sp. (T. Iso)
- B. *Chroomonas salina*
- C. *Thalassiosira pseudonana*
- D. *Chaetoceros calcitrans*
- E. *Dunaliella tertiolecta*
- F. *Pavlova lutheri*
- G. *Tetraselmis suecica*



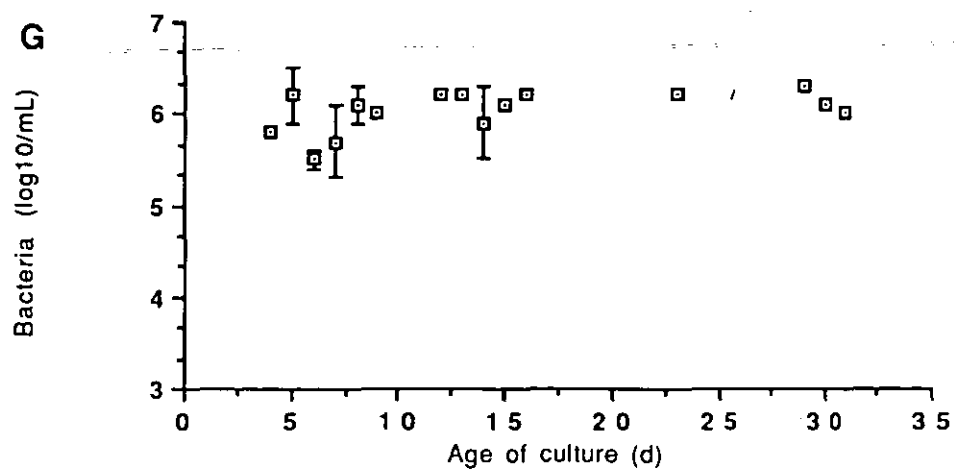
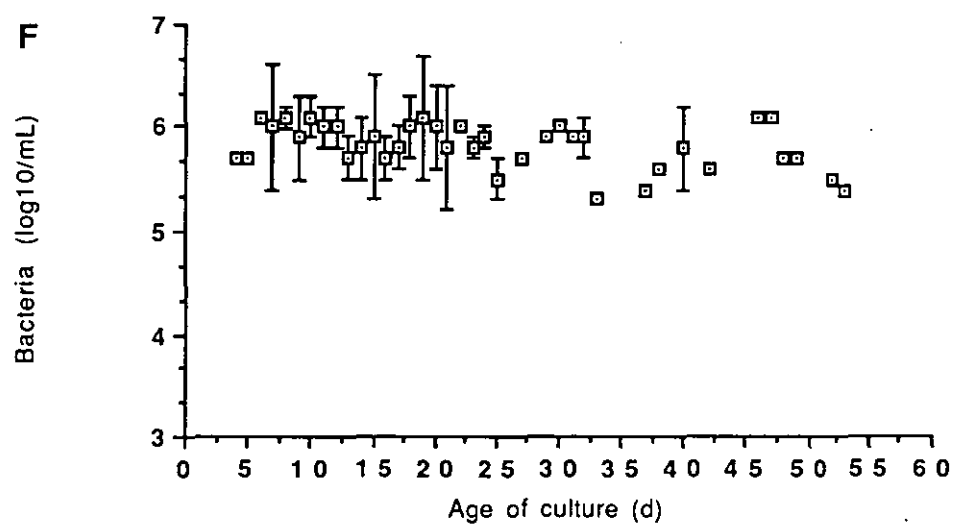
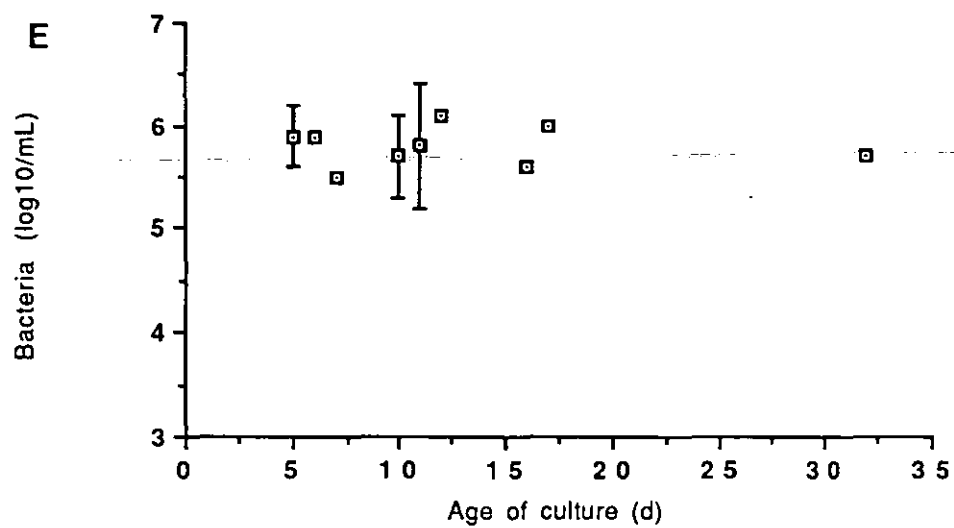


Table 18. Bacteria (mean  $\pm$  sd; SWAV) in microalgal cultures fed to larval oysters in 1985/86.

Microalgal clone <sup>a</sup>	5 L starter culture (N)	Bacteria ( $\log_{10}$ units mL <sup>-1</sup> )
T. Iso	A <sup>b</sup> (10)	5.7 $\pm$ 0.5
	N <sup>c</sup> (10)	5.6 $\pm$ 0.7
3H	A (20)	6.1 $\pm$ 0.4
	N (8)	6.1 $\pm$ 0.6
Pav	A (7)	5.8 $\pm$ 0.5
	N (3)	5.7 $\pm$ 0.9
Tet	A (2)	6.0 $\pm$ 0.8
	N (2)	6.1 $\pm$ 0.8

<sup>a</sup>: see Abbreviations, page 4.

<sup>b</sup>: Axenic.

<sup>c</sup>: Non-axenic.

been inadvertently contaminated during the sub-inoculation process. These cultures never contained presumptive Vibrionaceae (TCBS).

#### 4.0 Bacteriological Examination of Sick and Healthy Larvae.

Samples of sick (inactive) and healthy (active) larvae from the same batches were homogenised and the homogenate inoculated onto SWAV and TCBS plates.

Total viable (SWAV) results are shown in Figure 19. These show an increase in numbers of bacteria associated with larvae with increasing larval size. There is also a marked increase in the number of bacteria associated with sick larvae compared to healthy larvae.

Presumptive Vibrionaceae (TCBS) were only rarely found to be associated with larvae.

#### 5.0 Production of Oyster Larvae.

Figures for the production of ready-to-set oyster larvae during the 1985/86 production season (Table 17) represent an improvement on the previous season, with the survival rate of larvae increasing from 3.2% to 4.6%. The first ready-to-set animals were seen at similar times in the two seasons.

It was notable that only 1 of 8 batches of larvae was totally lost due to bacterial disease during this season. Small or major losses occurred in other batches, but less frequently than in 1984/85. Six of the 7 surviving batches grew at an even rate and the mean growth curve (not shown) was very similar to that in the earlier season (Figure 15).

#### D. Analysis of Factors Associated with Larval Mortalities.

Hatchery records for the 1984/85 and 1985/86 production seasons were reexamined with regard to the development of larval batches with which correlation with bacteriological data of the microalgal cultures fed to those larvae was possible.

Analysis of data for 18 of the 21 batches spawned during the 2 seasons indicated the existence of a level of viable (SWAV) bacteria in the microalgal cultures above which it was not safe to feed microalgae to oyster larvae. A bacterial level of  $\geq \log_{10} 6.3$  units  $\text{mL}^{-1}$  of feed microalgae culture was implicated in the development of bacterial disease by the larvae.

The impact of this bacterial level on the growth and survival of 4 larval batches is clearly shown in Figure 20. When microalgal cultures containing  $< \log_{10} 6.3$  bacteria  $\text{mL}^{-1}$  were fed to larvae (Figure 20A), the larvae grew satisfactorily to the ready-to-set stage without loss due to bacterial disease. (The only larvae discarded were those deliberately culled out for the purpose of density adjustment).

In contrast, when microalgal cultures with  $> \log_{10} 6.3$  bacteria  $\text{mL}^{-1}$  were fed to very young larvae, losses were sustained within a few days (Figures 20B-20D). If surviving larvae were then fed only microalgal cultures containing  $< \log_{10} 6.3$  bacteria  $\text{mL}^{-1}$  they grew satisfactorily (Figure 20B), but survival to the ready-to-set stage was mildly to moderately reduced. If, however, the surviving larvae were fed microalgal cultures containing  $> \log_{10} 6.3$  bacteria  $\text{mL}^{-1}$ , further losses occurred, resulting in a poor yield of ready-to-set larvae (Figure 20C) or none at all (Figure 20D).

Figure 19. Viable (SWAV) bacteria associated with active and inactive oyster larvae.

**Key.**

$\Delta$  active

■ inactive

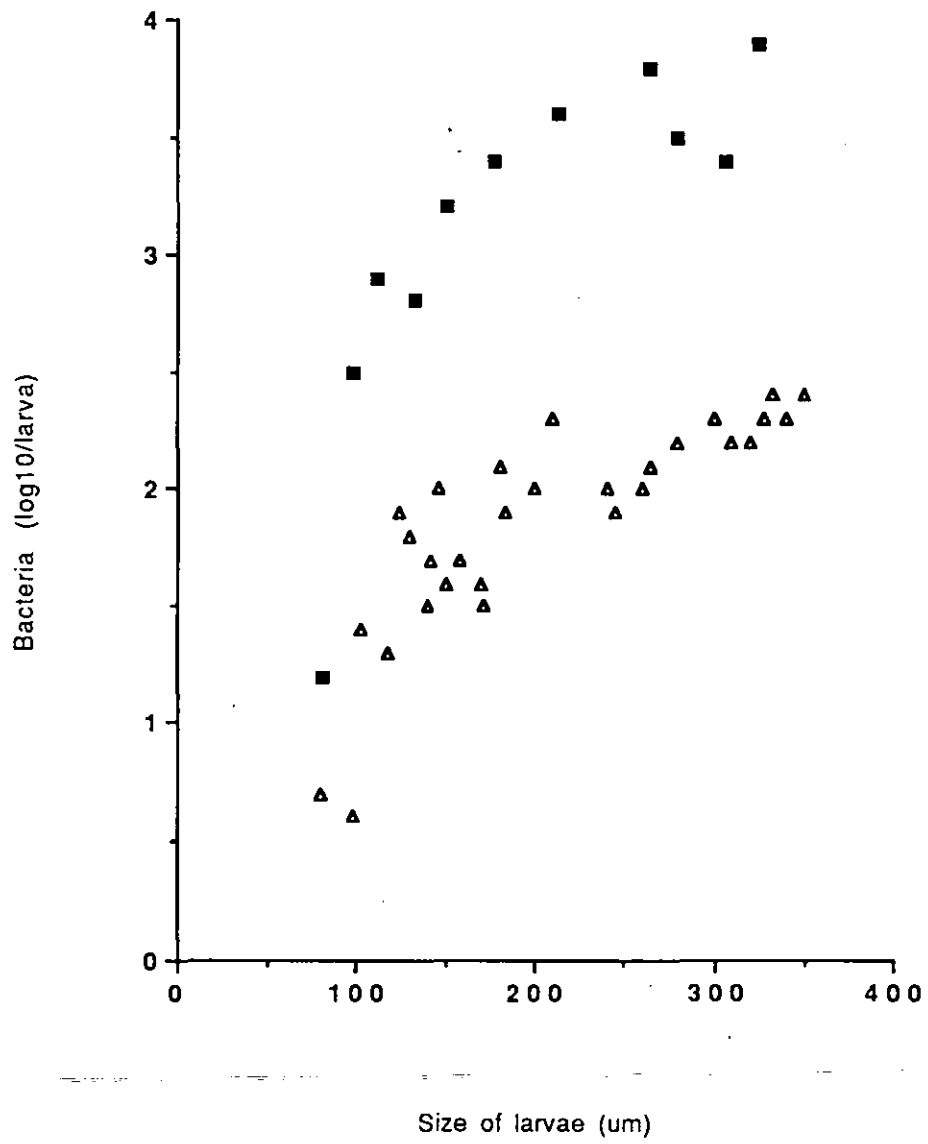




Figure 20. Growth curves of larval oysters (■).

The bacterial count (SWAV) in microalgal cultures fed to larvae on specific days is indicated by ( $\Delta$ )

The safe threshold of bacteria in microalgal cultures ( $\log_{10} 6.3 \text{ ml}^{-1}$ ) is indicated by (---)

Key.

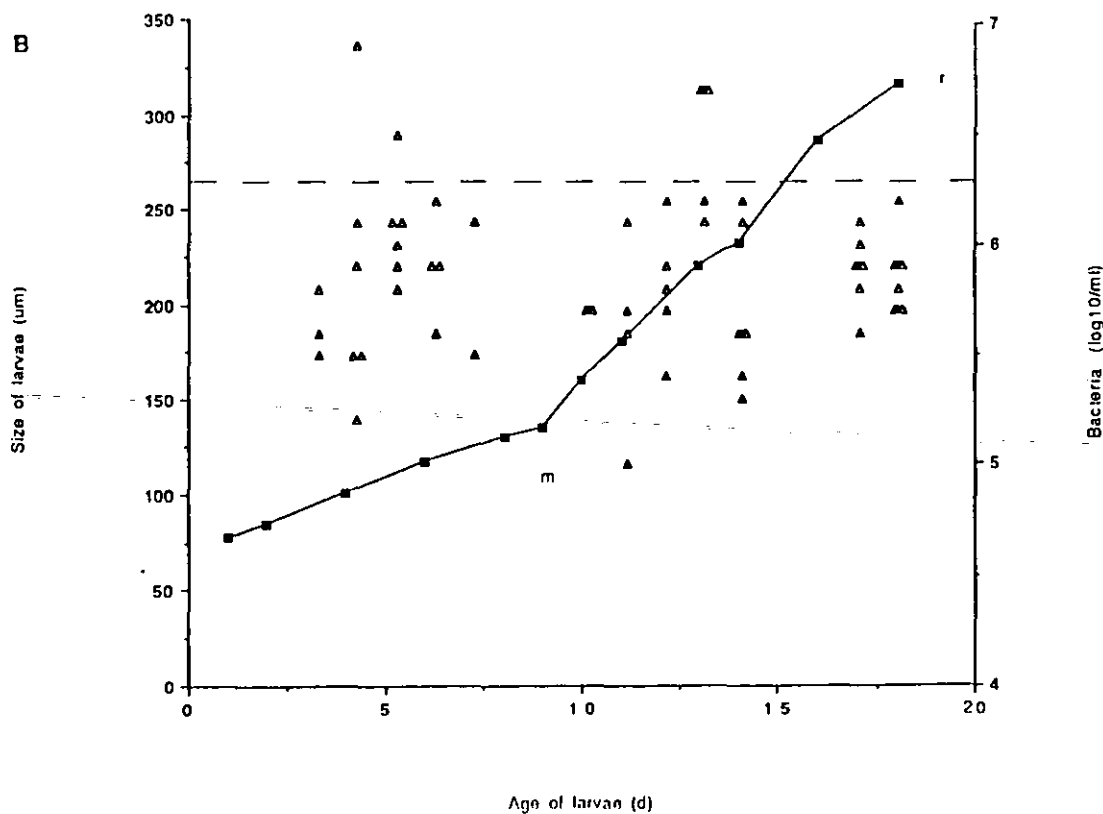
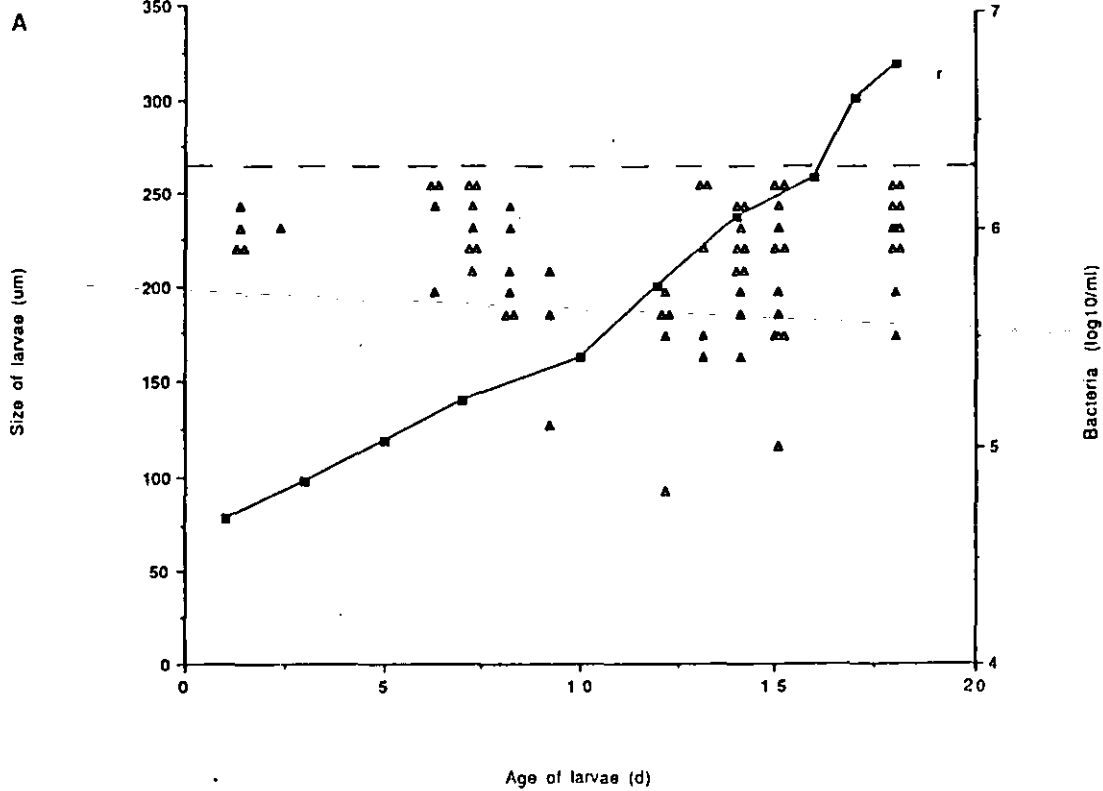
s = **small loss (15-25%) of larvae**

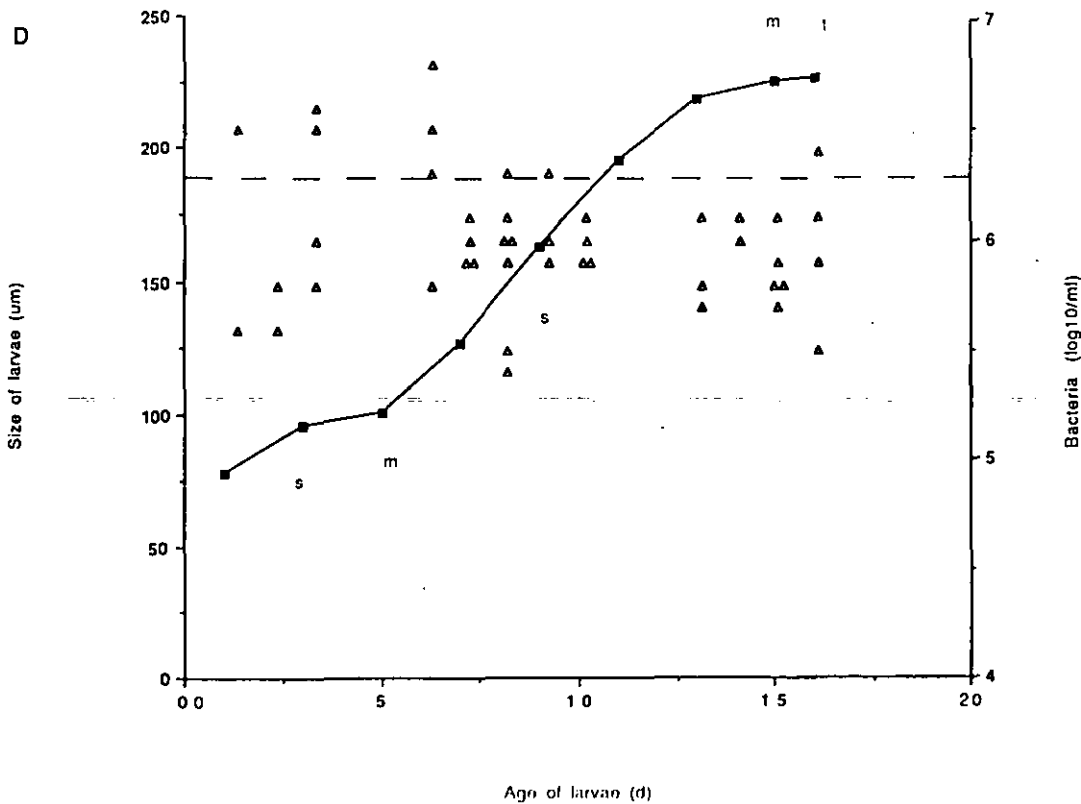
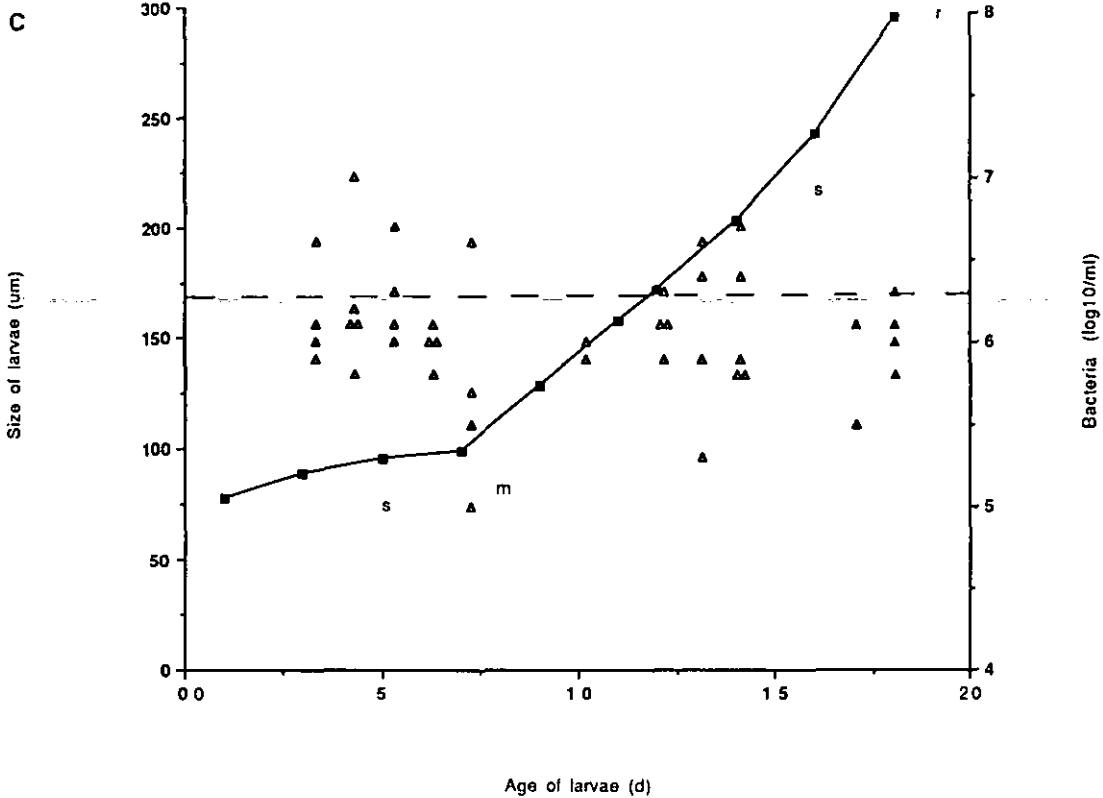
m = major loss (25-90%) of larvae

t = total loss (> 95%) of larvae

r = ready-to-set larvae.

see text for details





Further examination of the records revealed the following:

i) the feeding of microalgal cultures containing  $< \log_{10} 6.3$  bacteria  $\text{mL}^{-1}$  to 1 to 19+ day-old larvae was associated with no larval loss(es), and with the target yield of ready-to-set larvae (5% of fertilised eggs, eg. Figure 20A).

ii) the feeding of microalgal cultures containing  $> \log_{10} 6.3$  bacteria  $\text{mL}^{-1}$  to 1-7 day-old larvae was related almost immediately to a reduced growth rate (Figures 20B and 20C cf Figure 20A).

Small (15-25%) larval losses were sustained within 1-7 days, or major (25-90%) or total ( $> 90\%$ ) loss(es) within 1-5 days. These patterns are shown in Figures 20B-20D and were exhibited by batches 1984/85-7, -10 and -11, 1985/86-2, -3, -4 and -5 in Table 19.

If surviving animals were fed microalgal cultures containing  $< \log_{10} 6.3$  bacteria  $\text{mL}^{-1}$ , growth was satisfactory but with a moderately low yield of ready-to-set larvae (3-4.5% of fertilised eggs) as shown in Figure 20B (batch 1985/86-3 in Table 19).

However, if surviving animals, or  $\geq 8$  day old animals which had not sustained early losses, were fed microalgal cultures containing  $> \log_{10} 6.3$  bacteria  $\text{mL}^{-1}$ , small or major losses occurred within 1-7 days as shown in Figure 20C (batches 1984/85-7, 1984/85-8, 1984/85-10, -11 and -12, 1985/86-2 in Table 19), resulting in a poor yield of ready-to-set larvae (1.5-3% of fertilised eggs). Alternatively, major and total losses occurred within 1-4 days as shown in Figure 20D (batches 1984/85-4, 1985/86-4 in Table 19).

Table 19. Relationship between feed microalgae cultures containing > log<sub>10</sub> 6.3 viable (SWAV) bacteria mL<sup>-1</sup> and larval mortality.

Batch No.	Larval age (d)	Microalgal cultures with > log <sub>10</sub> 6.3 bacteria mL <sup>-1</sup> [clone <sup>a</sup> X <sup>b</sup> (N <sup>c</sup> )]	Larval mortality
84/85-4	12	3H 6.8 (15), C.cal 6.6 (15)	
	16		t
84/85-7	2	C.cal 7.1 (33), C.cal 6.5 (33)	
	3	C.cal 7.3 (25)	
	5	3H 6.8 (19)	s
	6	3H 6.6 (17)	
	7	3H 6.5 (11)	m
	13		s
	14	3H 6.4 (7)	
	16	3H 6.5 (9)	s
	17	3H 6.5 (9)	s
	18	3H 6.6 (25), T.Iso 6.7 (17)	
		Dun. 6.5 (17)	s
	19	Dun 6.5 (25)	s
84/85-8	9	3C 7.0 (14), C.cal 7.1 (14)	
	10	C.cal 6.5 (13)	
	11	T.Iso 6.6 (12)	
	12	3H 7.0 (10), Dun 6.6 (15)	
	14		s
	16		s

Table 19 continued.

84/85-10	3	C.cal 6.6 (25), C.cal 7.0 (25)	
	5		m
	6		m
	8	C.cal 6.5 (20), C.cal 6.7 (16)	
	9	C.cal 6.5 (20), C.cal 6.5 (16)	
		3H 6.5 (8), T.Iso 6.5 (12)	
	11	3C 6.5 (20)	
	12		s
	13		s
	15		m
	16	3H 6.6 (33), 3C 6.5 (33)	

---

84/85-11	1	C. cal 6.7 (100)	
	4	3H 6.8 (33)	s
	5	3H 7.0 (50)	s
	9		m
	16	Dun 6.4 (15)	
	18	3H 6.7 (60)	
	19	3H 6.8 (25)	s
	20	Dun 6.6 (25)	s

---

84/85-12	12	C.cal 6.5 (13), 3H 6.5 (13)	
		3H 6.5 (13)	
	14	3H 6.7 (21)	
	16		s
	17	3H 6.7 (20)	
	18	3H 6.7 (13)	
	19	3H 6.8 (18)	
	20	Dun 6.6 (5)	
	23	3H 6.6 (14)	
	24		s
	26		s

---

Table 19 continued.

85/86-2	3	3C 6.8 (14)	
(Fig	4	3C 7.0 (10)	
18C	5	3C 6.7 (22)	s
	6		m
	7	Pav 6.6 (20)	m
	9		s
	13	Pav 6.7 (22), T.Iso 6.4 (22)	
	14	T.Iso 6.7 (25)	
	17		s
<hr/>			
85/86-3	4	3C 6.9 (11)	
(Fig	5	3C 6.6 (16)	
18B)	9		m
<hr/>			
85/86-4		1	3C 6.5 (11)
(Fig	3	3H 6.5 (17)	
18D)	5		s
	6	3H 6.8 (15), T.Iso 6.5 (25)	
	7		m
	9		s
	15	3C 6.6 (20)	
	16	Tet 6.4 (20)	m
	17		t
<hr/>			
85/86-5	1	T.Iso 6.4 (33), Pav 6.7 (33)	
	2	T.Iso 6.7 (25)	
	5		m
	7		s
<hr/>			

<sup>a</sup>: See Abbreviations, page 4.

<sup>b</sup>: Viable (SWAV) bacteria in microalgae culture ( $\log_{10}$  units  $\text{mL}^{-1}$ ).

<sup>c</sup>: % (v/v) of diet fed on that day.

The hypothesis was that oyster larvae fed any microalgal culture containing  $>\log_{10} 6.3$  viable (SWAV) bacteria  $\text{mL}^{-1}$  would suffer loss/es,  $>10\%$ , due to disease, while larvae fed only microalgal cultures containing  $<\log_{10} 6.3$  viable (SWAV) bacteria  $\text{mL}^{-1}$  would suffer no such loss/es.

As data presented in this thesis (Figure 20, Table 19 and pages 143 and 147) support this hypothesis unequivocally, statistical analysis was not considered necessary.



iii) in all instances, the feeding of 2 (and often 3 or 4) specific cultures of feed microalgal cultures containing  $> \log_{10} 6.3$  bacteria  $\text{mL}^{-1}$  over one or more days was related to larval loss(es).

iv) all clones of feed microalgae were implicated in one or more larval loss(es). *C. calcitrans* and *T. pseudonana* 3H were implicated most often in 1984/85, while *Isochrysis* sp. and *Ch. salina* 3C were the major causes of bacteria-induced larval mortality in 1985/86.

v) the proportion which implicated clones formed of the daily diet varied greatly and thus did not allow prediction of the extent of larval loss(es). Similarly, the total numbers of viable (SWAV) bacteria in implicated cultures varied greatly and was also not predictive of the extent of larval mortality.

vi) the biotypes of bacteria in implicated cultures were highly varied, as indicated by diverse colonial morphologies and other growth characteristics. However, the biotypes very rarely included presumptive (TCBS)*Vibrio* spp., which were isolated from 3/93 (3.2%) samples in 1984/85 (mean count  $\log_{10} 2.66$  units  $\text{mL}^{-1}$ ) and 3/421 (0.7%) samples in 1985/86 (mean count  $\log_{10} 2.52$  units  $\text{mL}^{-1}$ ) and never included red-pigmented motile rods (see Brown 1974, 1981a).

vii) there was no seasonal influence on the number or type of larval losses experienced due to bacterial disease, with outbreaks occurring throughout the 1984/85 production season (Figure 21A) and sporadically in the early part of the 1985/86 production season (Figure 21B).<sup>a</sup>

<sup>a</sup> Please see facing page.

### **E. Direct (Light Microscopic) Counts of Bacteria in Feed Microalgal Cultures.**

The total number of viable (SWAV) bacteria in feed microalgae could only be determined retrospectively, after 6-8 days incubation on the agar plates. Because there was a great need to know if the bacterial count exceeded  $\log_{10} 6.3 \text{ units mL}^{-1}$  immediately prior to feeding the microalgae to larvae, a rapid, direct microscopic counting method was tested.

The direct count of bacteria in the microalgal cultures was always found to be higher than the viable count (Appendix 5). However, the correlation between the two counts was very variable, as indicated by the size of the standard deviations (Table 20). **Thus the direct method for enumerating bacteria in microalgal cultures did not provide a more rapid evaluation of safe microalgal cultures, and was therefore not pursued.**

It is also very clear from the figures given in Appendix 5 that there was a poor correlation between the viable or direct counts of bacteria in the microalgal cultures and the concentration of microalgal cells in the cultures at the time of harvest.

Figure 21. Monthly losses of larvae due to bacterial disease.


A. 1984/85 production season


B. 1985/86 production season


Key.

a. Start of production season

b. Finish of production season

small loss = 15-25% mortality 

major loss = 25-90% mortality 

total loss = > 90% mortality 

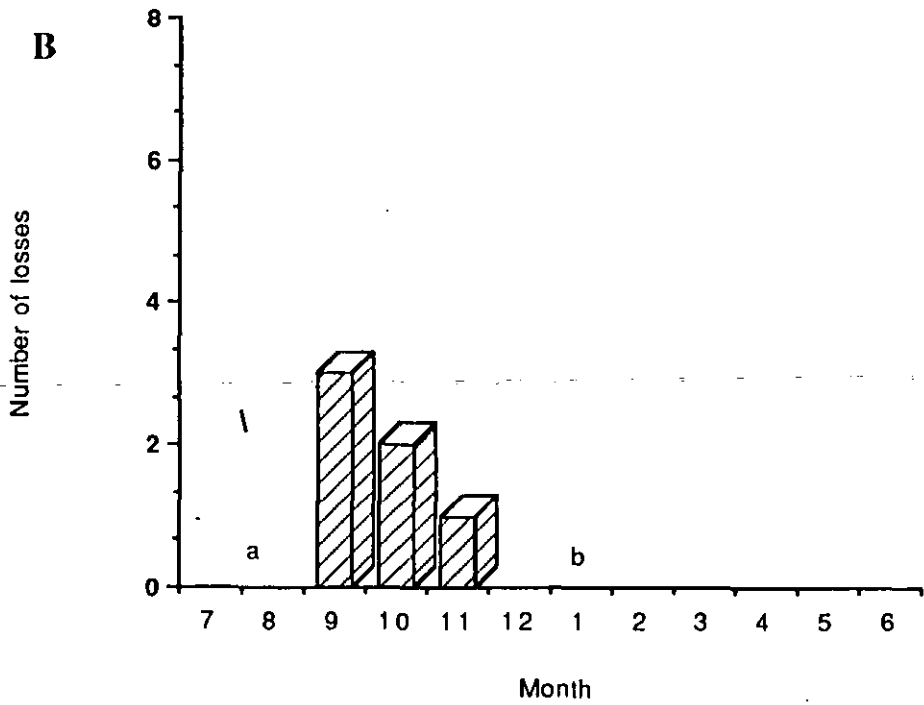
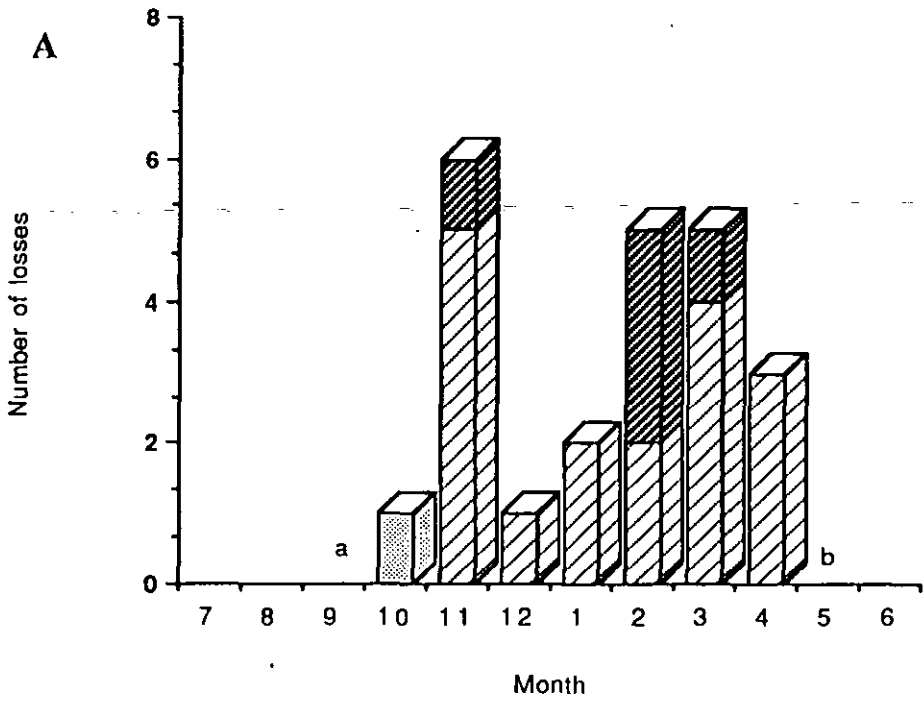


Table 20. Relationship between direct microscopic (D) and viable (spread-plate, SWAV).(V) methods for counting bacteria in **microalgal** cultures.

Microalgal clone <sup>a</sup>	D/V (mean $\pm$ sd; N=10)
T. Iso	7.1 $\pm$ 3.1
3C	3.1 $\pm$ 2.8
3H	6.7 $\pm$ 5.4
C. cal	6.2 $\pm$ 3.1
Dun	7.9 $\pm$ 4.3
Pav	6.9 $\pm$ 3.4
Tet	14.0 $\pm$ 10.0

<sup>a</sup>: see Abbreviations, page 4.

## **F. Other Bacteriological and Non-Bacteriological Factors Affecting Larval Survival.**

### **1.0 Introduction.**

Although it was shown that bacteria in microalgal cultures fed to oyster larvae were the major factor causing larval disease outbreaks, it was important to examine other factors which may have influenced larval survival rates during the 2 production seasons. These have been separated into bacteriological and non-bacteriological factors, as follows.

### **2.0 Bacteriological factors.**

#### **2.1 Removal of Biofilms from Internal Surfaces of Pipes, Hoses and Microalgal and Larvae Tanks**

The collection tank for harvested microalgae and all delivery pipes (both for seawater used as larval culture medium and harvested microalgal cultures) discharging into the larval tanks were either rinsed with hot (approx. 60°C) fresh water or dried thoroughly, with forced air, after each use. This treatment was effective in reducing the bacterial population colonising the internal surfaces of these fixtures to below that detectable by the swab technique (see Materials and Methods section B.1.2).

After each rearing period the inner surface of larvae tanks was mechanically cleaned as described in Materials and Methods section A.4.4.3. This cleaning method was also consistently effective in the reduction of the bacterial levels on the surfaces of the larvae tanks to below that detectable by the swab technique.

## 2.2 Bacteriological Quality of Intake Seawater.

As described in Materials and Methods section A.1, seawater for use within the hatchery was pumped through a sand filter, into a settling tank and from there into a header tank where the water was heated to 25-28°C during a holding period of 1 - 2 hours. The heated water was then delivered directly into the larvae tanks.

Parallel water samples, for bacteriological cultivation tests, were obtained from the following sites:

- a) the sea near the seaward end of the intake lines,
- b) the settlement tank
- c) the header tank and
- d) the larvae tanks (after filling but before the addition of any larvae or microalgal cultures).

Total (SWAV) counts (Table 21) increased significantly during the passage of water from the sea to the settlement tank and from the settlement tank to the header tank (Students t-test,  $P < 0.001$ ). This increase in numbers was due to the shedding into the intake seawater of bacteria colonising the immersed surfaces of the piping and biological filter, which for practical reasons were only cleaned once every 2-3 months. Such increases are common in plumbing systems (Characklis and Cooksey 1983).

There was no significant difference (Students t-test) between the total (SWAV) counts obtained from the header tank and the larvae tanks (Table

21). (This was because the delivery lines between the header tank and larvae tanks were regularly cleaned and dried as described previously. It is interesting to note that larvae tank water counts ( $3.6 \pm 0.3 \log_{10}$  units  $\text{ml}^{-1}$ ) were similar to the safe level  $< 3.90 \log_{10}$  units  $\text{ml}^{-1}$ ) described by Murchelano *et al.* (1975) at a hatchery for rearing *C. virginica* larvae at Long Island Sound, U.S.A.

There were no significant differences (Students t-test) in presumptive Vibrionaceae (TCBS) counts between any of the sample sites (Table 21), due to the high daily variation of the numbers of this group of bacteria in the various samples (Appendix 6).

During the period of this study it was noted that winds from a south to south-easterly direction caused sufficient wave action to stir up bottom sediment around the intake line. Analysis of both total (SWAV) and presumptive Vibrionaceae (TCBS) counts from the four sample sites (Appendix 6), showed no significant variations (Students t-test) in bacterial levels with respect to prevailing weather conditions.

### 2.3 Broodstock.

The process by which adult oysters were conditioned for spawning in the hatchery resulted in their shells becoming fouled with organic matter of both endogenous (faeces, pseudofaeces) and exogenous (detritus, dead microalgae cells etc.) origin. At the hatchery this has been regarded as a major potential source of bacterial contamination of eggs and larvae. Consequently, before each spawning attempt, all broodstock were carefully scrubbed and washed in order to minimise fouling. Thus the risk of bacterial infection of oyster larvae from this source was greatly reduced.



Table 21. Viable bacteria (SWAV, TCBS; mean  $\pm$  sd log<sub>10</sub> units mL<sup>-1</sup>) in seawater samples (n=36).

Culture Media	Oceanic seawater <sup>a</sup>	Settling Tank	Header Tank	Larval Tanks <sup>b</sup>
SWAV	2.9 $\pm$ 0.4	3.4 $\pm$ 0.3	3.7 $\pm$ 0.4	3.6 $\pm$ 0.3
TCBS	0.6 $\pm$ 0.7	0.6 $\pm$ 0.6	0.6 $\pm$ 0.7	0.6 $\pm$ 0.7

<sup>a</sup>: collected near the hatchery seawater intake line (see Figure 12).

<sup>b</sup>: before the addition of larvae or microalgal cultures.

## 2.4 Fertilised and Unfertilised eggs.

No viable (SWAV) bacteria were isolated from washed (see Materials and Methods section B.1.4) fertilised or unfertilised eggs. These data were supported by scanning electron microscopic examination of fertilised oyster eggs (Figure 22), showing them to be bacteria free. Thus the possibility of eggs and/or sperm acting as a vector of the bacterial diseases experienced at the hatchery could be discounted.

## 2.5 Larvae Tank Water.

Counts of viable (SWAV) and presumptive Vibrionaceae (TCBS) bacteria from larval tank water are shown in Table 22. There was a significant (Students t-test) rise in SWAV counts between water immediately after larvae and feed microalgae had been added and the counts after one or two day rearing periods ( $P < 0.001$ ). However, there was no significant difference (Students t-test) between the SWAV counts of water containing sick or healthy larvae after either one or two day rearing periods.

There were no significant changes in the level of presumptive Vibrionaceae in the larvae water during either one or two day rearing periods..

## 3.0 Non-Bacteriological Factors.

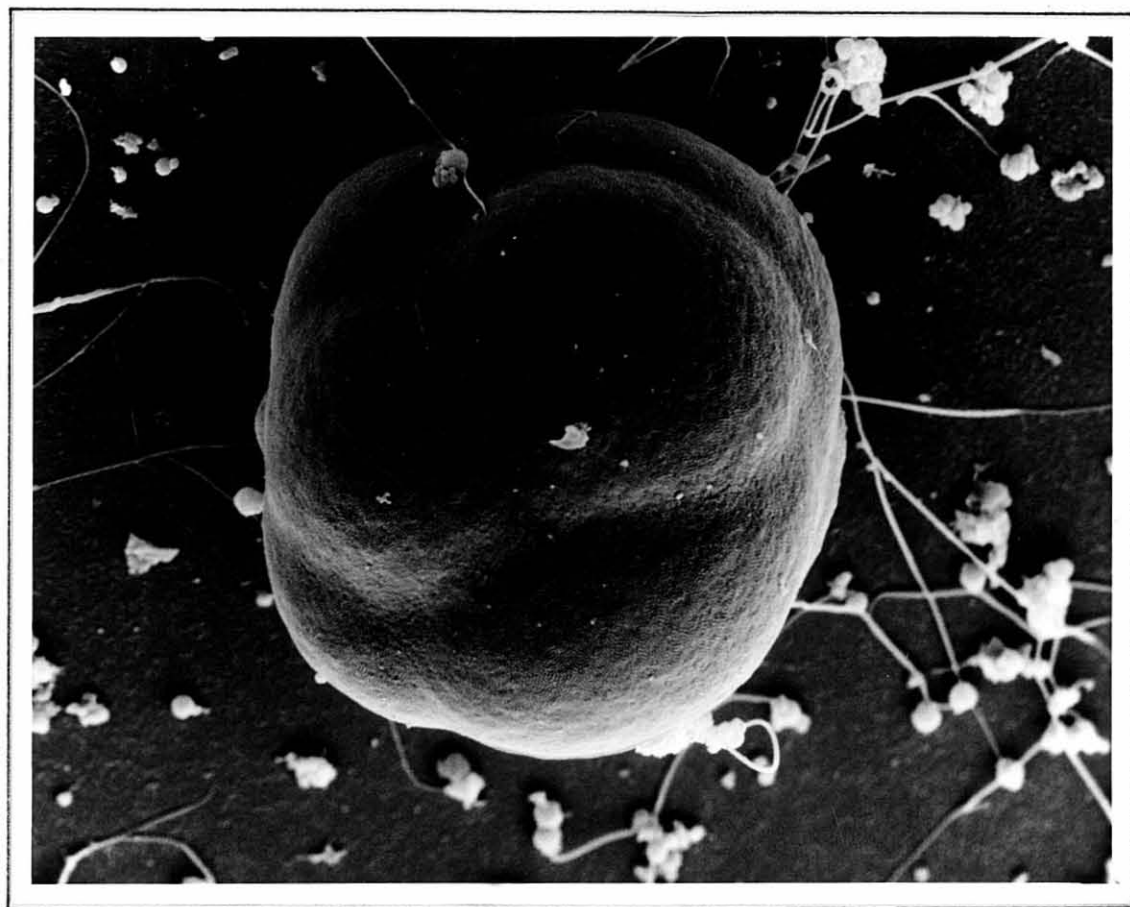
### 3.1 Water Temperature

In both the 1984/85 and 1985/86 production seasons larvae were reared at temperatures between 24 and 28°C. This temperature range was considered optimal for the rearing of this species (Loosanoff and Davis 1963).

Figure 22. Fertilised oyster egg.

Note absence of surface associated bacteria.

SEM x 3000, bar = 3  $\mu\text{m}$ .



3  $\mu$

### 3.2 Water Quality.

Physical parameters of water quality measured regularly were temperature, salinity and pH. Variations in ambient seawater temperature would not have effected the oyster larvae as the temperature of the larvae culture medium was always greater than ambient seawater temperatures. The density and pH of the intake water varied only slightly throughout this study (1020-1026  $\text{gmL}^{-1}$ ; 8.1-8.3 respectively) and showed no significant temporal variation. There were no significant variations of these factors between the three production seasons.

### 3.3 Broodstock.

In both seasons, adult oysters used as broodstock were obtained from a wide range of commercial oyster farms throughout Tasmania and conditioned in a similar manner in the hatchery. Eggs and sperm were observed microscopically, before fertilisation, to ensure they had no morphological defects. Consistently high quality eggs were obtained using this method, as evidenced by fertilisation rates always being greater than 95%.

### 3.4 Gene-Pool.

In each season, 50-90 animals were used for each spawning. This number of broodstock was sufficient to provide a >95% probability that recessive genes would not be expressed (Gosling 1982).

### 3.5 Larval Density.

The larval density guide-line (Table 8) was adhered to very closely in both the 1984/85 and 1985/86 production seasons. In 1983/84, however,

Table 22. Counts (mean  $\pm$  sd  $\log_{10}$  units  $\text{mL}^{-1}$ ) of viable (SWAV) and presumptive Vibrionaceae (TCBS) bacteria in larval tank water.

Length of rearing period (days),(N)	Health of larvae <sup>a</sup>	Bacterial count.....			
		SWAV initial <sup>b</sup>	SWAV final <sup>c</sup>	TCBS initial	TCBS final
1, (15)	H	3.8 $\pm$ 0.7	4.9 $\pm$ 0.5	1.0 $\pm$ 1.1	1.1 $\pm$ 0.7
1, (25)	S	3.2 $\pm$ 0.5	5.0 $\pm$ 0.6	0.8 $\pm$ 1.0	1.3 $\pm$ 1.1
2, (31)	H	4.0 $\pm$ 0.7	5.7 $\pm$ 0.5	1.1 $\pm$ 0.8	0.9 $\pm$ 0.7
2, (14)	S	3.8 $\pm$ 0.4	5.4 $\pm$ 0.3	1.2 $\pm$ 0.5	1.1 $\pm$ 0.6

<sup>a</sup>: at end of rearing period: H = healthy (active); S = sick (inactive).

<sup>b</sup>: immediately after addition of larvae and feed microalgae.

<sup>c</sup>: at end of rearing period.

densities were lower than the guide-lines due to the extensive larval losses caused by bacterial disease.

### 3.6 D-shape Veligers at 24 hours Post-Fertilisation.

The mean values for % transformation from eggs to D-shaped larvae, and the size of the 1 day old larvae were very similar for the two production seasons (i.e 1984/85: 64.6%, 79.6  $\mu\text{m}$ ; 1985/86: 67.7%, 78.2  $\mu\text{m}$ ). This is a very satisfactory transformation rate and is indicative of the use of high quality eggs and sperm.

### 3.7 Nutritional Requirements.

The mix of microalgal clones fed to larvae varied considerably between seasons, as new clones became available (Table 9). However, in each season a balanced diet of documented nutritional value (eg. see Langdon and Waldcock 1981; Gerdes 1983; Brown *et al.* 1989) was fed.

During the production seasons under investigation, all of the factors mentioned in Results sections F.3.1 to F.3.7 were controlled by appropriate husbandry practices. They were therefore not considered to have exerted any detrimental effects on larval survival. It was the considered opinion of hatchery management and staff that there was not sufficient variation between these husbandry practices and those of the 1983/84 production season (as determined from hatchery records) to account for the marked improvement in larval survival rates observed during this study (M. Fraser, pers. comm.)

## DISCUSSION

The primary aim of this study was to identify and suggest commercially acceptable methods to rectify the cause/s of massive mortalities of larval oysters (*Crassostrea gigas*) such as those which occurred at the private hatchery in Bicheno, Tasmania during its 1983/84 production season. Failure to find an economic solution to this problem would have led to financial collapse of the company.

Preliminary investigations, performed during the 1983/84 production season indicated that bacteria, pathogenic to oyster larvae, were the major cause of mortalities. This result was not surprising, as both the literature and anecdotal evidence from other mollusc hatcheries around the world highlight pathogenic bacteria as being the main factor limiting larval production in both experimental and commercial hatchery operations. For this reason a detailed bacteriological survey of the hatchery was conducted, concentrating on possible sources of bacteria in general and, specifically, sources of presumptive vibrionaceae, as this latter group has been implicated in many outbreaks of bacterial disease in cultured mollusc larvae.

Although some scanning electron microscopic examinations of microalgal cells were performed, the methods employed in the survey consisted primarily of the culture of viable bacteria on solid growth media. There have been criticisms of this technique of enumeration of bacteria, based on observations that all culture media are selective to some extent (Bell *et al.* 1974, Jones 1982) and that bacteria in some microalgal cultures tend to form aggregates (Murchelano and Brown 1969, Seki 1971). Furthermore, estimates of the proportion of bacteria in seawater samples which are capable of growth on solid media vary considerably, ranging from 0.0001% in deep oceanic water (Tabor *et al.* 1981) to about



10% in coastal environments (Jannasch and Jones 1959, Kogure *et al.* 1984). There is also a problem with using direct microscopic counts of bacterial cells as an indication of the number of actively respiring bacteria in seawater samples. Meyer-Reil (1978) and Zimmerman *et al.* (1978), using different techniques to estimate the proportion of respiring cells in seawater samples, came up with figures of 2.3-56% and 6-12% respectively. Thus it seems that no single technique will give an accurate estimate of the total number of active bacterial cells in a given sample.

For the purposes of this study, it was necessary to gain estimates of the level of viable bacteria in liquids and on surfaces, using a counting method which did not require specialised equipment or complex, time-consuming techniques. Low cost and ease of operation were important prerequisites, as the procedures developed were to be used by hatchery personnel after the present study was completed. Consequently, it was decided to employ a standard viable-counting technique using a solid bacteriological culture medium. The medium selected for this study had previously been shown to promote the growth of a consistent proportion of bacteria from marine samples (Lewis *et al.* 1985).

Factors influencing the bacteriological quality of growth medium used for semi-continuous culture of microalgae at the Bicheno hatchery were identified as follows:

- a. the aseptic techniques employed during set-up and normal operation of the semi-continuous harvest/refill sequence for the 500 L microalgal cultures.
- b. the bacteriological quality of the seawater used as growth medium for the 500 L microalgal cultures.

Aseptic techniques used by hatchery staff during preparation of 500 L microalgal culture bags for inoculation, the inoculation of microalgal starter culture into the 500 L bags, harvesting the feed microalgae and refilling the bags with growth medium were found to be satisfactory. There was very little chance for air- or surface-borne bacterial contamination of the 500 L cultures during any of these activities. The procedures for these stages of the microalgal culture process remained unchanged during the course of this study.

It appears that pasteurised seawater is bacteriologically unsatisfactory for use as growth medium for microalgae if pasteurisation occurs at a distance from the microalgae culture room. It was impractical to sterilise lengthy seawater delivery lines and heat exchange tubes such as those used in the hatchery during the 1983/84 production season. Without sterilisation, the inside surfaces of the delivery lines rapidly became colonised by bacteria which either survived the heat treatment or contaminated the pasteurised seawater during the cooling period prior to delivery to the culture bags. Those bacteria colonising the wet (inner) surfaces of the pasteurised seawater delivery pipes were presumably surrounded by a glycocalyx matrix (Costerton *et al.* 1981a). This matrix would have acted to partially protect the bacteria closest to the pipe surface against thermal shock from further passages of warm seawater, thus allowing those bacteria to multiply steadily between treatments of the plumbing system with disinfectant. The method used to disinfect the delivery lines was also probably only partially effective as no mechanical cleaning of the surfaces was possible. Glycocalyx matrices have been shown to be very effective barriers against antibacterial compounds. They are able to protect a proportion of the bacteria in a matrix from the effects of antibacterials, thus leaving a residual population to recolonise a surface

rapidly when conditions allow (Costerton *et al.* 1981a,b, Characklis and Cooksey 1983).

The rapid rise in bacterial numbers in the pasteurised seawater while still in the pasteurisation tanks and after delivery to the 500 L bags was presumably due to a combination of bacteria shedding from bacterial films on the inside surfaces of the tanks and plumbing and, to a lesser degree, multiplication of these bacteria once in the water column (Costerton *et al.* 1981a, Characklis and Cooksey 1983). Increases of the magnitude found during the cooling process in this system are typical of those encountered in freshly stored seawater. Zobell (1946) noted that counts of culturable bacteria in seawater, stored in non-metallic containers, increased rapidly within the first 24 hours after collection. The rate of increase was proportional to the area of wet surface in the containers. Murchelano *et al.* (1975) recorded 10- to 100-fold increases in the viable counts of bacteria in seawater stored in sterile tanks during the initial 24 hours of storage, but no significant increases between 24 and 48 hours after collection.

High bacterial levels in the microalgal growth medium during the 1983/84 production season were thought to be the major factor contributing to the excessive bacterial numbers found in the feed microalgal cultures. The hatchery manager also highlighted the need to develop a more efficient method by which to produce growth medium for the feed microalgae, as the pasteurisation-cooling system was very time consuming and expensive to operate. Therefore the system for disinfecting the growth medium was revised prior to the start of the 1984/85 production season.

The use of 0.2  $\mu\text{m}$  membrane-filtration technology for the production of growth medium for the 500 L cultures of feed microalgae immediately offered tangible benefits for hatchery operations. Growth medium prepared in this way could be produced on demand, with no delay while

the pasteurised seawater cooled. Bacteriological benefits were also obvious. The growth medium could be delivered directly to each 500 L culture bag through a short length of flexible hose, which could be sterilised (autoclaved) after use each day. Thus the problems of bacteriological contamination of the growth medium inherent in the pasteurisation-cooling system were eliminated.

The presence of viable bacteria in the 0.2  $\mu\text{m}$  filtrate was not surprising, considering the number of reports of filterable and dwarf bacteria present in natural marine waters (Anderson and Heffernan 1965, Meyer-Reil 1978, Tabor *et al.* 1981, Torrella and Morita 1981, MacDonell and Hood 1982, Li and Dickie 1985). The result which was initially surprising was the similarity in numbers of viable (SWAV) bacteria removed from the 0.2  $\mu\text{m}$  filtrate by 0.2 and 0.1  $\mu\text{m}$  pore size membrane filter-discs. This can be explained by considering the pressure differentials across the membranes of the growth medium filtration system (120 kPa) and the filter-discs (approximately 30 kPa). Small bacterial cells in the seawater, slightly larger than 0.2  $\mu\text{m}$  in diameter, could have been forced through the former membrane under high pressure, while the pressure of the latter system was not sufficient to allow passage of the bacteria through the 0.2  $\mu\text{m}$  filter-discs.

Suggestions by Li and Dickie (1985) that bacterial cells passing through a 0.2  $\mu\text{m}$  filter can be later retained on a 0.2  $\mu\text{m}$  membrane disc because they either increase in size, form microcolonies or are aspherical in shape do not appear valid in this case. A period of several hours would be needed for dwarf cells to increase in size or form microcolonies, in response to a possible increase in nutrients released by the destruction of other cells on the filter membrane (Kogure *et al.* 1984). All samples in the present study were processed within 30 minutes of collection, thus allowing no time for either dwarf cells to increase in size or aggregates to

form. It is also unlikely that asymmetry of bacterial cells can explain the apparent inconsistency. The counts of bacteria collected on the 0.2 and 0.1  $\mu\text{m}$  pore size filter-discs were too similar to be attributed to random passage of correctly aligned cells through the disc-filter membranes.

The very low numbers of viable bacteria in the 0.2  $\mu\text{m}$  membrane-filtered growth medium were considered satisfactory for the culture of microalgae as food for oyster larvae. It was not considered advisable to produce a sterile growth medium, as lack of competition would have allowed, at any stage after the bags had been filled, the rapid proliferation of <sup>single or limited species of</sup> bacterial contaminants. A small diverse bacterial population, such as that presumably present in 0.2  $\mu\text{m}$  membrane-filtered growth medium, was considered to be ecologically more stable than a bacteria free environment. Similar observations regarding the creation of sterile growth media for mass culture of marine invertebrates and feed microalgal cultures were made by Murchelano *et al.* (1975).

The presence of low levels of bacteria in the membrane-filtered microalgal growth medium led to some biofouling problems. The filtration system, including the flexible delivery hose, was full of growth medium for up to 10 hours each day, and as such a bacterial film formed on the inside surfaces of all components. Semi-quantitative analysis of the number of bacteria colonising the surfaces indicated that the rate of biofilm formation was proportional to the numbers of bacteria in the seawater adjacent to the surfaces and the length of time the surfaces were in contact with the water. It was for this reason, as well as to avoid possible cross-contamination between culture bags, that any new bags were filled as soon as the growth medium membrane-filtration system had been set up each day. Any bags from which microalgae had been harvested were refilled only after all new bags had been filled.

The poly-propylene components of the first 0.2  $\mu\text{m}$  membrane-filtration system, used during the 1984/85 production season, were unable to withstand the temperatures associated with autoclaving or steaming and therefore had to be cleaned by hand. Incomplete disinfection of these components by hatchery staff, which occurred occasionally, resulted in the overnight survival of some bacteria in the biofilms attached to the filter components. When the system was assembled each day, any surviving bacteria recolonised the wet surfaces of the filter components faster than if the surfaces had been properly disinfected. Thus, bacteria would start shedding into the growth medium earlier and at a faster rate than normal. The inability to routinely achieve total disinfection of the filter components resulted in the production of growth medium with variable bacteriological quality.

This problem was exacerbated by the periodic breakdown of the membrane in the 0.2  $\mu\text{m}$  filter cartridges. It was not possible, when relying on bacteriological data for indications of membrane integrity, to be certain the cartridge to be used on any day was functioning properly. Bacteriological results reflecting the integrity of the membrane in a particular cartridge were only available retrospectively, up to 7 days after the cartridge had been last used.

To reduce the incidence of filter breakdown the type of 0.2  $\mu\text{m}$  cartridges used was changed to a model designed to withstand repeated autoclaving. Loss of integrity of the filter membranes in the new cartridges was very rare. The vast majority of these cartridges were discarded only after repeated usage had caused the membrane to clog to an extent where flow-rate through the filter was decreased to below a practical level.

The final modification to the membrane-filtration system, made prior to the start of the 1985/86 production system, was the acquisition of an

autoclavable housing for the 0.2  $\mu\text{m}$  filter cartridges. The housing was fitted with attachments which permitted aseptic, *in situ* integrity testing (bubble-point method, Brock 1983) of the filter membrane. This allowed the entire 0.2  $\mu\text{m}$  membrane-filtration unit and delivery hose to be assembled and autoclaved as one unit and the integrity of the filter membrane to be tested immediately prior to use each day. Any cartridge found to be faulty was discarded before it was used and a sterile replacement cartridge installed aseptically in the housing. Using this system, the initial integrity of the 0.2  $\mu\text{m}$  cartridge to be used was assured. Therefore the possibility of the growth medium being delivered to the 500 L bags being of sub-standard bacteriological quality was reduced to a minimum.

The overall mean level of viable (SWAV) bacteria in the 0.2  $\mu\text{m}$  membrane-filtered growth medium during 1985/86 was very similar to that of the previous season. However, as indicated by the smaller standard deviation of the mean, the variability of the counts in 1985/86 was lower than in 1984/85. The decrease in variability can be attributed to the use of an autoclavable filter housing and the *in situ* integrity testing of the filter membranes.

It is evident from the results that the incorporation of a 0.2  $\mu\text{m}$  membrane-filtration system for the production of growth medium for semi-continuous microalgal culture resulted in significant improvements with respect to the time required to prepare the growth medium and its bacteriological quality.

Growth characteristics of bacterial populations in 500 L cultures of feed microalgae examined during this study were all very similar. Low initial levels of bacteria rose rapidly during the first 6-8 days until reaching a plateau level which was maintained for up to 13 weeks. Similar patterns of bacterial growth in microalgal cultures have been reported by

Bell *et al.* (1974). Possible explanations for the maintenance of plateau levels of bacterial numbers in microalgal cultures are discussed below.

Two mechanisms for the limitation of the numbers of bacteria associated with both natural and experimental microalgal blooms have been described. The first is whereby bacterial activity is limited by the availability of appropriate nutrients. Bacterial uptake of dissolved organic matter (DOM) released by phytoplankton is very efficient. Bell (1984) found that, under low-nutrient conditions, bacteria show a high substrate affinity, but low specificity, to DOM released by microalgae. Bacterial activity under these conditions is therefore limited by the availability of the substrate. Rapid utilisation of DOM by bacteria in microalgal cultures acts to keep the level of DOM to a minimum (Derenbach and Williams 1974, Larsson and Hagstrom 1979, Wolter 1982), thus prolonging the nutrient-poor conditions (as experienced by the heterotrophic bacteria) and linking bacterial production directly to the rate of release of algal extracellular products.

Stability of microalgal cell numbers and thus DOM production rates (Burney *et al.* 1982) is the major factor contributing to the stability of the number of respiring bacterial cells in bacteriovore-free microalgal communities (Larson and Hagstrom 1979, Fuhrman *et al.* 1980, Jensen 1983).

The second mechanism by which the size of a bacterial population in a microalgal culture can be limited was discussed by Bell (1983). The author presented preliminary evidence that prolonged exposure to a single alga may favour the development of bacterial populations in which metabolism is limited by enzyme-mediated transport of the compound/s available in the DOM pool. Although this would not be a significant factor in natural systems it is possible that such a situation could occur in algal monocultures as exist in many mollusc hatcheries.



The results of the current study do not establish clearly whether the plateau levels of bacteria in the feed microalgal cultures were determined by substrate- or transport- limited growth of the bacteria. However, it is likely that substrate availability was the prime factor limiting bacterial populations, as bacterial numbers in individual microalgal cultures tended to plateau even before the first harvest of those cultures. This indicates a decline in the amount of nutrients available to the rapidly increasing bacterial population.

The rate at which bacteria are able to assimilate available DOM would lead to a plateau in numbers only if the rate at which bacteria were being removed from the culture (e.g. by harvesting the microalgae) and bacterial replication rates in the culture were equal. Clearly this cannot be the case in microalgal cultures before the first harvest, or in cultures which were not continually harvested at a constant rate. Uneven harvest rates were the norm for cultures of feed microalgae at the Bicheno hatchery. Therefore the hypothesis of transport-limited bacterial replication rates can be discounted.

The three major factors which appeared capable of influencing the size of bacterial populations in 500 L cultures of feed microalgae grown at the Bicheno hatchery are as follows:

- a. the use of axenic or non-axenic starter cultures to inoculate the 500 L bags;
- b. the species of microalgae being cultured;
- c. the bacteriological quality of the growth medium used to fill the bag before inoculation with microalgae and refill the bag after harvest of a volume of microalgal culture.

The use of axenic, in place of non-axenic, starter cultures did not appear to influence the number of bacteria in the 500 L microalgal culture at harvest. During the 1985/86 production season both axenic and non-axenic starter cultures were used to inoculate 500 L bags with microalgae. No significant difference in the bacterial levels of harvested feed microalgal cultures due to the bacteriological quality of the starter cultures was evident. Nonetheless, the use of axenic starter cultures is recommended, as only then can the possibility of inoculating specific bacteria pathogenic to oyster larvae (e.g. *Vibrio tubiashii*, see Hada *et al.* 1984) or microalgae (Cole 1982, Guillard 1983) into the feed microalgal cultures be eliminated.

The species of microalgae being grown in the 500 L culture bags did not affect greatly the mean level of bacteria in cultures at harvest. There was little evidence that a given clone would support a greater or lesser bacterial population than any other clone. An exception to this general rule was apparent in cultures of *C. calcitrans*. Bacterial counts in 500 L cultures of this clone appeared to keep rising above the plateau levels reached by the other clones. However, as only young (1-2 week old) cultures of this clone were fed to larvae during the 1984/85 and 1985/86 production seasons, the levels of viable (SWAV) bacteria in the cultures were significantly lower than in cultures grown using pasteurised-cooled growth medium. The low rate of utilisation of this clone as food for larvae was a result of judgements of the quality of the cultures by hatchery staff. It was often found that, one or two weeks after inoculating a 500 L bag with this clone, the cells started to aggregate and the culture was discarded. During studies of declining microalgal blooms, Fukami *et al.* (1981a,b, 1983a) reported similar aggregations of senescent microalgal cells coincident with increases in the number of associated bacteria.

The factor which had the greatest impact on the overall bacteriological quality of the feed microalgal cultures was the level, and perhaps taxonomic diversity, of viable (SWAV) bacteria in the growth medium. Cultures of microalgae grown using 0.2  $\mu\text{m}$  membrane-filtered seawater as growth medium were found to contain significantly lower levels of planktonic bacteria than those grown using pasteurised-cooled seawater. There was also a lower incidence of bacterial colonisation of microalgal cells in microalgal cultures grown using membrane-filtered growth medium. There are two possible explanations for the lower plateau levels of planktonic bacteria in feed microalgal cultures grown in membrane-filtered growth medium as compared to those grown in pasteurised-cooled growth medium.

The first possibility is that those bacteria present in 0.2  $\mu\text{m}$  membrane-filtered growth medium had a lower affinity for DOM produced by the microalgae than the bacteria in the pasteurised-cooled growth medium. This is possible as, although no taxonomic studies were undertaken, it is likely that the taxonomic diversity of the bacteria in the two growth media was very different.

For this argument to be valid, bacteria in 0.2  $\mu\text{m}$  membrane-filtered seawater would need to have a lower affinity for the available DOM than the bacteria in the pasteurised-cooled growth medium. However, it has been shown that bacteria in low-nutrient conditions, such as those in the 0.2  $\mu\text{m}$  membrane-filtered seawater, have a high general affinity for microalgal DOM, and that marine bacteria in general have similar affinities for a wide range of microalgal extracellular products (Bell *et al.* 1974, Bell 1984). It would therefore be expected that bacteria in membrane-filtered growth medium would be able to utilise DOM released by microalgae in 500 L bags at a similar, or even slightly higher, rate to those bacteria adapted to the copiotrophic conditions existing within the

biofilms coating the inner surfaces of pasteurised-cooled seawater delivery pipes.

The second, and more likely, explanation for the observed difference in bacterial plateau levels in 500 L cultures of microalgae grown using the two growth media is that microalgal cultures grown in membrane-filtered growth medium contained lower levels of nutrients available to the bacteria. It has been reported that microalgae grown under sub-optimal conditions release a greater proportion of DOM than healthy microalgal populations (Bell *et al.* 1974, Sharp 1977, Fukami *et al.* 1981b, 1983a, Bratback and Thingstad 1985). As discussed above, bacterial activity in microalgal cultures is limited by the availability of DOM produced by microalgae. It therefore follows that healthy microalgal cultures will support a lower level of bacteria than cultures containing a large proportion of unhealthy microalgal cells. Senescent cells also show an increased tendency to be colonised by bacteria (Cole 1982, Kogure *et al.* 1982a) as they appear to produce lower amounts of antibacterial compounds (Bell *et al.* 1974).

All microalgal cultures grown using pasteurised-cooled growth medium (1983/84 production season) were exposed to high bacterial numbers during their entire culture cycle. All flask cultures were contaminated with bacteria, and the 5 L microalgal starter cultures were inoculated into growth medium containing high (approximately  $10^4 \text{ mL}^{-1}$ ) levels of viable (SWAV) bacteria. It is likely that the large bacterial populations associated with the microalgal cultures would have created stressful conditions for the microalgal cells, due to competition for nutrients (Rhee 1972, Parker *et al.* 1975, Currie and Kalf 1984) and/or the presence of bacteria pathogenic to microalgae (Berland *et al.* 1972, Cole 1982).

Another observation which indicates that the decreased bacterial plateau levels can be attributed to increased health of the cultures, rather than a change in the ability of bacteria in the growth medium to utilise microalgal DOM, is that not all clones showed a decline in numbers of associated bacteria when membrane-filtered growth medium was used. If declines in the plateau levels of bacteria in the microalgal cultures were due to a lower affinity of the bacteria in the membrane-filtered growth medium for DOM released by the microalgae, it would be expected that plateau levels in cultures of all clones would have dropped. This was not so. Bacterial plateau levels in 500 L cultures of *Ch. salina* and *C. calcitrans* were not significantly affected by the use of 0.2  $\mu\text{m}$  membrane-filtered seawater as growth medium. Thus it appears that, for these clones, culture conditions were not improved sufficiently, by the use of membrane-filtered growth medium, to result in a decrease in the production of extracellular microalgal DOM. Other modifications to culture techniques are obviously necessary for the health of cultures of these clones to be improved significantly.

It is also possible that some degree of algal antibiosis acted to limit bacterial levels in the 500 L microalgal cultures. Scanning electron microscopic examination of all clones <sup>provided evidence of</sup> only a small proportion of cells colonised by bacteria. This indicates the production of extracellular compounds by the microalgae which inhibit the attachment of bacteria. Those microalgal cells found to be colonised by bacteria were thought to be unhealthy or senescent (Droop and Elston 1966, Fukami *et al.* 1981a,b, Cole 1982, Kogure *et al.* 1982a). Although production of antibacterial compounds by marine phytoplankton has been documented (Duff *et al.* 1966) it is unlikely that these compounds would reach sufficient

concentrations in natural systems to affect significantly the numbers of bacteria in the surrounding water column. However, in closed systems such as the high density culture of microalgae, antibacterial compounds may reach levels whereby they influence the numbers and taxonomic diversity of planktonic bacteria (Bell *et al.* 1974).

This possibility also fits the hypothesis that microalgae cultured in this hatchery using membrane-filtered seawater are generally more healthy than those grown in pasteurised-cooled seawater. As mentioned above, healthy microalgal cells usually produce more antibiotic compounds and will therefore exert a greater limiting effect on bacterial growth rates than unhealthy cells. It is likely, however, that long-term exposure to a fixed pool of antibiotics, such as would occur in a microalgal monoculture, would result in increased resistance of the bacteria to those compounds. If this occurred, a steady increase in the bacterial population would be expected until it was again limited by some environmental factor. Unfortunately the microalgal cultures used during this study were not maintained for sufficient time for this hypothesis to be pursued.

The fact that some cells in all clones of microalgae cultured in membrane-filtered growth medium <sup>except *P. lutheri*</sup> were colonised by bacteria indicates that culture conditions were still, to some degree, sub-optimal.<sup>a</sup> Whether this was caused by the presence of bacteria pathogenic to the microalgae or by unfavourable environmental factors (e.g. light intensity or duration, nutrient availability, temperature, harvest intensity) is unclear. However, it remains that new technology, developed during this study, resulted in a significant increase in the efficiency of microalgal production in this hatchery. Investigations into measures to improve further microalgal culture conditions were the next obvious step in this process. However research into such improvements was not within the scope of this study.

<sup>a</sup> In this case sub-optimal conditions are defined as those predisposing microalgal cells to bacterial colonisation.

It was clear from hatchery production figures that a significant improvement in larval survival had been achieved during the 1984/85 and 1985/86 production seasons. Analysis of bacteriological and non-bacteriological factors relevant to the production of larvae at the hatchery indicated that the reduction of the plateau levels of viable (SWAV) bacterial levels in the 500 L feed microalgal cultures was responsible for the dramatic reduction in larval mortality rates. Epidemiological evidence suggests that microalgal cultures containing  $> \log_{10} 6.3$  bacteria  $\text{mL}^{-1}$  were the principal vectors of disease. Other authors have also suggested that feed microalgal cultures may be a potential source of bacteria pathogenic to bivalve larvae (DiSalvo *et al.* 1978, Guillard 1983, Elston 1984). However, these reports considered only the possibility of microalgal cultures harbouring populations of specific larval pathogens. The potential effects of the total bacterial population in microalgal cultures on larvae were not discussed.

Toxicity trials for extracellular compounds produced by two *Vibrio* strains (Brown 1983) indicated that levels of exotoxin, equivalent to approximately  $10^4$  viable bacterial cells  $\text{L}^{-1}$  larval culture medium, elicited disease in D-shape oyster (*C. virginica*) larvae. When toxin levels reached the equivalent of  $10^8$  viable bacterial cells  $\text{L}^{-1}$  larval culture medium, teratogenic effects on fertilised eggs were seen. Brown (1974) found that a minimum of  $10^3$  viable cells of a marine pseudomonad  $\text{L}^{-1}$  larval culture medium inhibited the development of *M. mercenaria* larvae.

These estimates of the number of bacteria required to elicit signs of disease in bivalve larvae are much lower than those found at the Bicheno oyster hatchery. However, it should be noted that Brown (1973, 1984) used pure cultures of known bivalve pathogens and established that these known pathogens produce toxins at low cell concentrations. In the case of the mixed populations of bacteria in the feed microalgal cultures in the

present study, it is suggested that toxicity associated with microalgal cultures occurs only after the critical level of  $\log_{10} 6.3$  bacteria  $\text{mL}^{-1}$  is reached.

Generally, the first indication that a batch of larvae was unhealthy was the appearance of patches of inactive larvae on the bottom of one or more larvae tanks. Microscopic examination of the affected larvae, collected either from the bottom of the tank or from the water column, revealed varying proportions of larvae showing a range of abnormalities, from pale internal organs, indicating poor feeding, to visceral and velar necrosis, to the total dissolution of body tissues. Bacteria "swarming" around moribund larvae (Guillard 1959, Tubiash *et al.* 1965, Brown and Losee 1978, DiSalvo *et al.* 1978, Brown 1981a, Tubiash and Otto 1986) were seen only rarely, although low levels of swarming bacteria may have passed undetected. Swarming bacteria are usually a sign of advanced bacterial disease (Brown 1983). Unless an epizootic occurred very rapidly, unhealthy larvae were discarded by hatchery staff before this stage of disease was reached.

The mechanism by which feed microalgal cultures containing  $> \log_{10} 6.3$  viable (SWAV) bacteria  $\text{mL}^{-1}$  elicit disease in oyster larvae was not examined. However, the course of disease in larval oysters at the Bicheno hatchery was very similar to pathogenesis types II and III as described by Elston and Liebovitz (1980). Both these types of disease are caused by extracellular production of toxins by bacteria.

The following observations also indicate that larval mortalities were caused by exposure to bacterial metabolites which were toxic to larvae at low concentrations.



- a. all clones of microalgae were implicated in one or more larval losses.
- b. neither the concentration of viable (SWAV) bacteria in the implicated cultures nor the total volumes of these cultures fed to the larvae were related to the severity of ensuing disease.

Typical volumes of microalgal cultures fed to batches of larvae at the Bicheno hatchery varied between 50 and 150 L. Therefore, when a feed microalgal culture, containing a "safe" bacterial level of  $10^6$  viable (SWAV) bacteria  $\text{mL}^{-1}$ , was pumped to a batch of larvae, in a 10,000 L larvae tank, the larvae would have been exposed to an additional  $5 \times 10^6$  -  $1.5 \times 10^7$  bacteria  $\text{L}^{-1}$  of larval culture medium. The addition of the same volume of a microalgal culture with the minimum suggested "unsafe" bacterial level of  $2 \times 10^6$  viable (SWAV) bacteria  $\text{mL}^{-1}$  would only double the number of bacteria added to the larvae culture media but nonetheless would be expected to result in some or all of the larvae becoming affected. Results gained during this study suggest the distinction between bacteriologically safe and unsafe feed microalgal cultures was very fine. A relatively small increase in the bacterial population in a bacteriologically "safe" microalgal culture could render it "unsafe" to feed to oyster larvae. This indicates the presence of toxic compound/s in "unsafe" microalgal cultures. Whether toxins were produced in microalgal cultures only after the critical level of bacteria was exceeded, or whether the concentration of the toxins increased dramatically as bacterial numbers passed this level is unclear.

It is also possible that direct ingestion of bacteria originating from the "unsafe" microalgal cultures, especially those bacteria colonising unhealthy microalgal cells, could result in the onset of disease. However, this is very unlikely to be the case, as senescent microalgal cells would have been present in all cultures and thus would have been ingested by larvae in all batches. If ingestion of senescent cells was a cause of larval mortalities, then such mortalities would have occurred in all batches of larvae and not just in those fed microalgal cultures containing  $> \log_{10} 6.3$  viable (SWAV) bacteria  $\text{mL}^{-1}$ .

Thus it would appear that larval mortalities observed during this study were the result of toxic metabolites released by bacteria in the feed microalgal cultures. Invasion of the internal organs of sick larvae by bacteria appeared only to occur after the disease had caused the larvae to become inactive, as described by Elston and Liebovitz (1980) and Brown (1983). The difference in the size of the populations of viable (SWAV) bacteria associated with active and inactive larvae (Figure 19) also indicates that bacterial invasion of larval tissues only occurred after the larvae become moribund.

The methods used to estimate numbers of viable (SWAV, TCBS) bacteria associated with sick (inactive) and healthy (active) oyster larvae may have resulted in an underestimation of the true values, due to the possible attachment of some bacteria to particles of homogenised larvae. However, the shear forces exerted on attached bacteria during the initial vigorous homogenisation of the larvae and mixing of the dilution series would have resulted in the vast majority of any irreversibly attached bacteria being dislodged into the water column (Dawson *et al.* 1981, Hermansson and Marshall 1988).

The total number of viable (SWAV) bacteria associated with healthy larvae is positively correlated to the size of the larvae. Garland *et al.*

(1982) reported the absence of surface-associated microorganisms from the internal organs of healthy, actively feeding, adult oysters. The authors suggested the major mechanisms restricting microbial growth within oysters are ciliary movement and mucous secretion (i.e. the mechanisms involved in the normal entrapment and mobilisation of food particles). If these mechanisms are also present in larval oysters, then bacteria associated with healthy oyster larvae must only be present either on the surface of the larval shell and/or attached to food particles ingested by the larvae.

Numbers of bacteria (SWAV) associated with sick (inactive) oyster larvae are much greater than those associated with healthy larvae, with differences of  $\log_{10}$  1-1.5 units being common (Figure 19). There are two possible explanations for this phenomenon. Firstly, it may reflect an increase in the number of bacteria colonising the outer layers of the larval shells, with no increase in the level of internal bacterial colonisation. The second alternative is that the moribund larvae lose their ability to prevent bacteria from colonising their internal organs and that such colonisation occurs, perhaps in conjunction with an increase of the levels of bacteria attached to the shell. Elston and Liebovitz (1980) demonstrated that the feeding activity of inactive larvae is greatly decreased. This would limit the antibacterial actions associated with normal feeding (Mori *et al.* 1980, Garland *et al.* 1982) resulting in an increase in the rate of bacterial colonisation of internal tissue.

The latter explanation for increased bacterial numbers associated with inactive larvae appears to be more likely, as bacterial invasion of the internal organs of moribund larvae of other bivalve species has been widely reported (Tubiash *et al.* 1965, Brown 1974, Elston and Liebovitz 1980, Elston 1982, Brown 1983).

*Vibrio* spp. have been widely implicated as causative agents of bacterial disease in bivalve larvae in this (Garland *et al.* 1983) and other (Tubiash 1975, Brown and Losee 1978, DiSalvo *et al.* 1978, Elston 1979, Brown 1981b, Elston *et al.* 1981) bivalve hatcheries. However, levels of presumptive Vibrionaceae associated with sick and healthy larvae enumerated during this study were always very low and very variable. There was no evidence that vibriosis posed a threat to the larvae during any stage of the production cycle.

## CONCLUSION

Practical and economic considerations preclude the provision of a bacteria-free environment for commercial culture of larval oysters. It was therefore the aim of this study to develop a procedural system whereby safe levels of bacteria in different areas of the hatchery operation could be defined, and bacterial populations could be easily kept below the defined level. It was also necessary to design the system to allow simple, routine bacteriological monitoring to be carried out by hatchery staff. Thus if a problem arose it could be quickly identified and corrected.

The monitoring system used in the hatchery can be likened to that developed to ensure microbiological safety and quality of prepared food products viz: the hazard analysis critical control point (HACCP) concept (ICMSF 1988). This concept provides a systematic approach to microbiological hazard identification, assessment and control by the following steps.

1. Identification of hazards and assessment of the potential magnitude of these hazards and the likely occurrence of a hazard associated with the various steps involved in the culture of oyster larvae.
2. Determination of critical control points (CCPs) at which identified hazards can be controlled. A CCP is a location, practice, or procedure which, if controlled, could minimise or prevent a hazard. Two types of CCP are identified: CCP1 that will ensure total control of the hazard; CCP2 that will minimise but cannot ensure the total control of a hazard.

3. Specification of criteria (e.g. levels of bacteriological contamination) that indicate whether an operation is under control at a particular CCP.
4. Establishment and implementation of procedure/s to monitor each CCP to check that it is under control.
5. Taking whatever corrective measures are necessary when the monitoring results indicate that a CCP is not under control.

The results of bacteriological studies of the oyster hatchery during two production seasons have allowed the major vector of bacteria pathogenic to oyster larvae to be identified. Following the HACCP system, the CCPs for the oyster hatchery are defined as shown in Figure 23.

At present the method used to monitor the bacteriological quality of the CCPs is the enumeration of viable bacteria on solid culture media. This method is slow, taking up to 7 days for a result to be known, and is unlikely to reveal the true numbers of viable bacteria present in any sample. However, reliable reference values for bacterial levels at the CCPs have been established using this system. Another positive consideration is that the bacteriological techniques used can be performed quickly each day by relatively inexperienced personnel with inexpensive equipment; a major consideration for small hatchery operations.

The strength of the procedures developed during this study is that, when performed correctly, they ensure:

- a. maintenance of hygienic conditions in which to culture oyster larvae,
- b. production of a high proportion of bacteriologically safe cultures of microalgae with which to feed the larvae.

During the development of these procedures compromises have been made between maintenance of stringent microbiological standards and economic considerations. Additional expense, in the form of extra equipment and increased labour costs, which would be required to further improve bacteriological standards in the hatchery, could not be justified from a commercial perspective. However, although the total elimination of bacterial disease in oyster larvae cultured at the hatchery has not been achieved, production of larvae has increased to a point whereby the hatchery company is financially self-supporting.

Figure 23. Hazard Analysis Critical Control Point (HACCP)  
diagram for the control of bacterial pathogens  
at the Bicheno oyster hatchery.

Key.

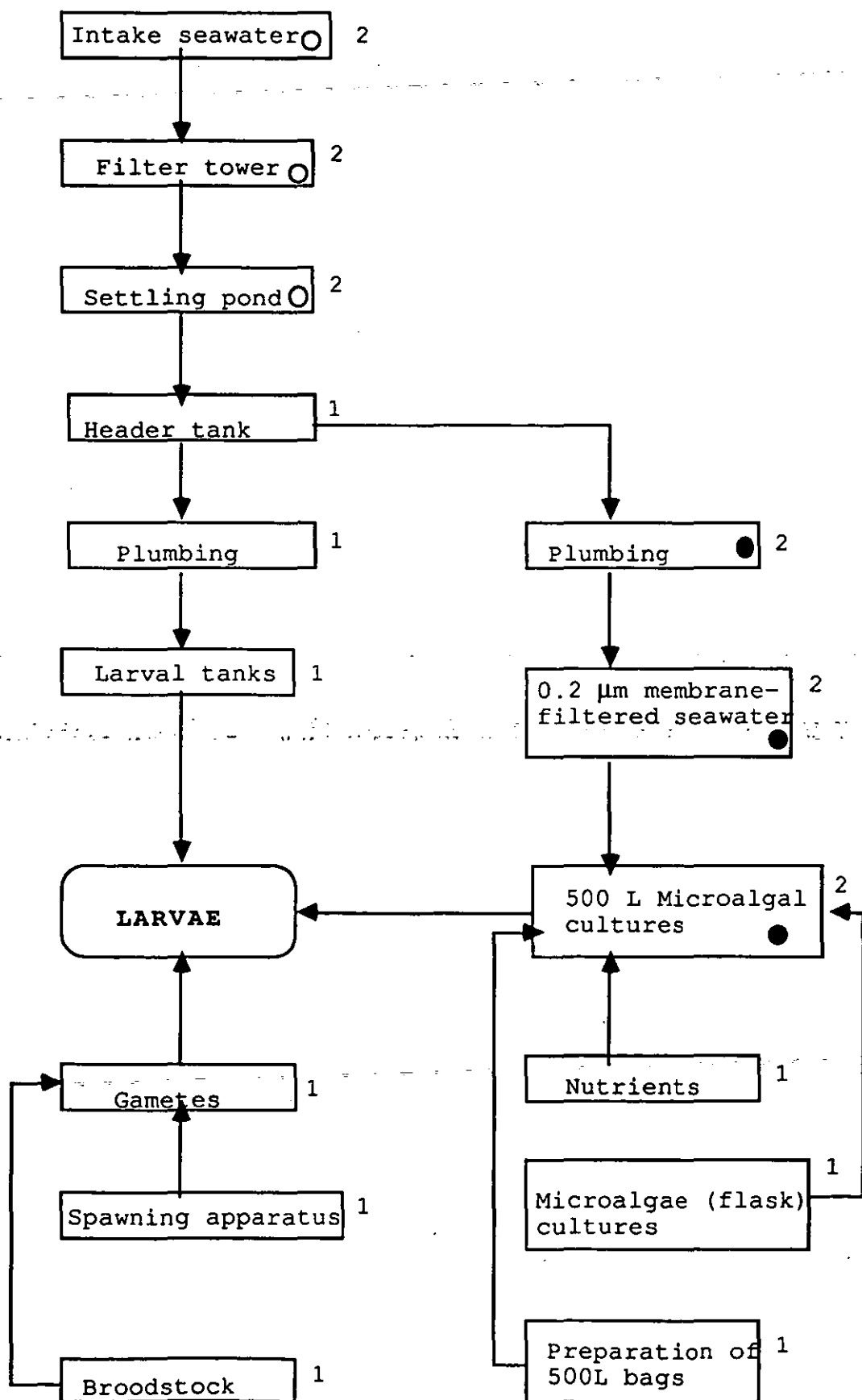
(○) indicates a site of minor contamination

(●) indicates a site of major contamination

1 indicates a CCP offering effective control  
of bacteria pathogenic to oyster larvae.

2 indicates a CCP offering partial control of  
bacteria pathogenic to oyster larvae.





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## APPENDICES

## Appendix 1. Microalgal Culture Medium f/2 (Guillard 1975).

This medium was prepared using A.R. or Technical Grade reagents. All stock solutions were autoclaved ( $110^{\circ}\text{C}$ , 30 minutes) immediately after preparation.

## Stock Solutions.

1. $\text{NaNO}_3$	$75 \text{ gL}^{-1}$
2. $\text{NaH}_2\text{PO}_4$	$50 \text{ gL}^{-1}$
3. $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$	$4.4 \text{ gL}^{-1}$
$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	$3.2 \text{ gL}^{-1}$
4. Trace metals	
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	$10 \text{ mgL}^{-1}$
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	$22 \text{ mgL}^{-1}$
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	$10 \text{ mgL}^{-1}$
$\text{MnCl}_2 \cdot 6\text{H}_2\text{O}$	$180 \text{ mgL}^{-1}$
$\text{NaMnO}_4 \cdot 2\text{H}_2\text{O}$	$6 \text{ mgL}^{-1}$
5. $\text{Na}_2\text{SiO}_3 \cdot 5\text{H}_2\text{O}$	$23 \text{ gL}^{-1}$
6. Vitamins	
Thiamin HCl	$22 \text{ mg } 100\text{mL}^{-1}$
Biotin	$0.1 \text{ mg } 100\text{mL}^{-1}$
Cyanocobalamin	$0.1 \text{ mg } 100\text{mL}^{-1}$

To prepare medium f/2.

Add 1 mL of each stock solution  $\text{L}^{-1}$  seawater. Autoclave prepared growth medium when possible.

## Appendix 2. Bacteriological Culture Media.

All media were autoclaved at 121°C for 15 minutes unless otherwise stated.

All chemical reagents were A.R. grade.

A vitamin solution was added to some media to enhance the viability of isolates (Lewis *et al.* 1985).

### 2.1 Filtered seawater.

Seawater was collected from the header tank after it had been filtered through a 25 µm mesh sieve.

### 2.2 Vitamin solution.

Thiamin HCl (Sigma)	0.1 mg
Biotin (Sigma)	0.5 µg
Vitamin B12 (Sigma)	0.5 µg
Deionized water	1000 mL

### 2.3 Sea Water Agar with Vitamins (SWAV).

Yeast extract (Oxoid)	1 gm
Bacteriological peptone (Oxoid)	1 gm
Filtered seawater (Appendix 1.1)	1000 mL
Vitamin solution (Appendix 1.2)	5 mL
Agar (Davis, grade J)	15 gm

## Appendix 2 continued.

## 2.4 TCBS Cholera agar.

TCBS (Thiosulphate-Citrate-Bile salt-Sucrose)

Cholera agar (Oxoid) 88 gm

Filtered seawater (Appendix 1.1) 750 mL

Deionized water 250 mL

Boil for 5-10 minutes then pour onto plates.

## 2.5 Plate Count Agar (PCA)

Plate count agar (Oxoid) 17.5 gm

Deionized water 1000 mL

### Appendix 3. Electron microscopy reagents.

All chemicals used were A.R grade.

#### 3.1 Marine salts solution.

NaCl	2.75 gm
MgCl <sub>2</sub>	1.05 gm
CaCl <sub>2</sub>	0.15 gm
Deionized water	100 mL
Autoclave at 121 <sup>0</sup> C for 15 minutes.	

#### 3.2 20% Formaldehyde solution.

Paraformaldehyde (Ajax)	2 gm
Deionized water	8 mL
1 M NaOH	5 drops

Heat suspension to 60<sup>0</sup>C until solid dissolves. Cool quickly and use within 2 hours of preparation.

#### 3.3 Combined aldehyde fixative.

20% (w/v) Formaldehyde (Appendix 3.1)	15 mL
25% Glutaraldehyde (Taab)	12 mL
1 M Sodium Cacodylate (Sodium dimethylarsinate-2.5 H <sub>2</sub> O) (Sigma)	10 mL
Marine salts solution (appendix 3.1)	60 mL
Deionized water	3 mL



Appendix 4. Counts of viable (SWAV) bacteria in 0.2  $\mu\text{m}$   
 membrane-filtered seawater collected on sterile  
 0.1 and 0.2  $\mu\text{m}$  (pore size) membrane discs.

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Collection	.....Bacteria 100mL <sup>-1</sup> .....	
time	0.1 $\mu\text{m}$ disc	0.2 $\mu\text{m}$ disc

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Morning	14	12
	8	10
	6	5
	0	0
	5	5
	12	10
	2	4
	0	1
	0	0
	7	8

Afternoon	60	49
	27	31
	10	11
	30	32
	15	13
	0	3
	10	8
	8	7
	21	23
	18	15

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Appendix 5. Counts of ( $10^6 \times N \text{ mL}^{-1}$ ) bacteria and microalgae cells in filtered ( $3\mu\text{m}$  membrane disc) and unfiltered feed microalgal cultures.

Key.

A = Viable (SWAV) bacteria in filtered microalgae cultures.

B = Viable (SWAV) bacteria in unfiltered microalgae cultures.

C = Direct (light microscopic) count of bacteria in filtered microalgal cultures.

D = Direct (light microscopic) count of microalgae cells in unfiltered microalgal cultures.

E = Ratio of direct bacteria count : viable bacteria count.

1. *Isochrysis* sp. (Tahitian clone)

	A	B	C	D	E
	1.6	1.3	12.2	3.1	7.6
	0.6	0.8	6.2	4.8	9.8
	1.0	0.8	2.3	3.2	2.3
	2.0	1.3	7.2	2.8	3.6
	0.5	0.4	5.1	2.9	10.2
	1.7	1.6	18.0	3.7	10.6
	1.0	1.2	10.1	2.6	10.1
	0.9	1.1	3.2	3.7	3.6
	0.6	0.4	4.1	2.1	6.8
	1.1	1.0	6.7	2.0	6.1
mean	1.1	1.0	7.5	3.1	7.1
sd	0.5	0.4	4.8	0.8	3.1

## Appendix 5 continued.

*2. Chaetoceros calcitrans*

	A	B	C	D	E
	0.3	0.4	1.5	5.6	5.0
	2.0	1.9	20.2	2.2	10.1
	0.6	0.8	3.1	4.8	5.2
	1.0	0.8	7.4	7.9	7.4
	0.8	1.0	3.9	8.5	4.9
	2.3	1.8	12.1	5.8	5.3
	0.9	0.7	0.5	5.0	0.6
	1.1	1.3	5.3	6.0	4.8
	0.9	1.3	6.9	7.2	7.7
	0.8	0.8	9.2	2.7	11.5
mean	1.1	1.1	7.0	5.6	6.2
sd	0.6	0.5	5.8	2.0	3.1

*3. Thalassiosira pseudonana*

	A	B	C	D	E
	1.0	0.9	9.1	5.5	9.1
	0.8	0.9	3.8	4.0	4.8
	0.6	0.8	1.2	2.6	2.0
	0.5	0.4	5.6	3.5	11.2
	0.5	0.4	8.3	2.5	16.6
	0.9	1.1	2.3	3.3	2.6
	1.1	0.9	7.4	4.1	6.7
	1.2	1.5	1.1	3.0	0.9
	0.8	1.2	9.7	5.1	12.1
	2.0	1.5	2.2	4.6	1.1
mean	0.9	1.0	5.1	3.8	6.7
sd	0.4	0.4	3.4	1.0	5.4

## Appendix 5. continued

4. *Chroomonas salina* 3C

	A	B	C	D	E
	1.0	1.1	3.8	0.5	3.8
	0.8	2.0	2.1	1.5	2.6
	0.5	0.4	1.2	2.1	2.4
	1.6	1.4	1.4	1.3	0.9
	1.0	0.8	2.5	0.8	2.5
	0.8	0.7	0.5	0.7	0.6
	0.7	0.7	0.5	1.8	0.7
	1.0	1.2	5.4	1.2	5.4
	0.5	0.5	1.3	0.7	2.6
	0.7	0.5	6.9	0.3	9.9
mean	0.9	0.9	2.6	1.1	3.1
sd	0.3	0.5	2.2	0.6	2.8

5. *Dunaliella tertiolecta*

	A	B	C	D	E
	1.6	1.4	10.6	1.5	6.6
	1.0	0.8	2.1	1.3	2.1
	0.8	0.7	8.4	0.4	10.5
	2.0	2.1	10.9	0.9	5.5
	1.0	1.0	15.1	0.9	15.1
	0.5	0.7	5.3	1.9	10.6
	0.9	0.7	5.7	1.0	6.3
	2.1	1.8	4.8	0.7	2.3
	1.5	1.1	11.2	1.0	7.5
	0.6	0.9	7.8	1.1	13.0
mean	1.2	1.1	8.2	1.1	7.9
sd	0.6	0.5	3.8	0.4	4.3

## Appendix 5 continued.

6. *Tetraselmis suecica*

	A	B	C	D	E
	0.5	0.7	9.1	0.2	18.2
	0.8	0.7	4.7	0.5	5.9
	1.0	1.2	22.1	0.9	22.1
	2.0	1.5	15.9	1.2	8.0
	1.8	1.4	3.9	1.5	2.2
	0.8	1.1	9.9	0.4	12.4
	1.2	1.1	11.4	2.4	9.5
	0.9	0.8	33.2	1.5	36.9
	0.9	0.9	8.7	0.6	9.7
	0.6	0.6	9.3	0.9	15.5
mean	1.1	1.0	12.8	1.0	14.0
sd	0.5	0.3	8.9	0.7	10.0

7. *Pavlova lutheri*

	A	B	C	D	E
	2.1	1.8	3.2	3.1	1.5
	0.7	1.0	4.9	2.1	7.0
	0.6	0.8	4.2	2.7	7.0
	0.9	1.0	8.9	3.4	9.9
	1.1	0.8	4.1	4.5	3.7
	1.5	1.5	10.1	2.7	6.7
	0.8	0.6	10.0	2.7	12.5
	2.0	1.8	7.9	2.5	4.0
	0.9	1.1	5.0	1.9	5.6
	0.7	0.5	7.7	1.4	11.0
mean	1.1	1.1	6.6	2.7	6.9
sd	0.5	0.5	2.6	0.9	3.4

Appendix 6. Viable bacteria in parallel seawater samples taken  
between 1 August and 30 September 1985  
(log<sub>10</sub> units mL<sup>-1</sup>)

.....Site <sup>a</sup> .....							
Ocean		Settling tank		Header tank		Larvae tanks	
SWAV	TCBS	SWAV	TCBS	SWAV	TCBS	SWAV	TCBS
2.8	1	3.5	0	3.9	0	3.8	1
2.2	0	3.2	0	3.7	0	3.6	1.2
2.4	1.5	3.6	0	3.5	1.2	3.5	0
2.7	1.3	3.8	0	3.3	0	3.9	0
2.9	0	3.2	0	3.4	0	3.1	1
3.0	0	3.1	1	3.6	0	3.8	1
2.9	0	3.8	0	3.1	0	4.0	0
2.9	0	3.4	0	3.5	0	3.5	0
2.8	1	3.6	1.2	3.7	1	3.6	0
2.7	1	3.6	1	4.2	1	3.1	1.5
2.8	0	3.0	1	4.3	2	3.3	1.3
2.9	0	3.4	0	3.7	0	3.6	1.2

Appendix 6 continued.

2.7	0	3.5	1.3	4.0	1	3.7	0	1
3.0	0	3.2	1.5	3.6	0	3.0	1	1
3.1	1	3.6	1	4.0	0	3.5	0	1
2.8	0	3.7	1	3.5	1.5	3.7	0	1
3.1	0	2.9	0	4.8	0	3.4	1	9
2.7	1.5	3.6	1	4.5	0	3.9	2	
2.6	0	3.7	1	4.0	1.4	3.8	0	9
2.8	0	3.4	1.4	4.5	1	3.5	1.2	1
3.8	0	3.9	0	3.7	0	4.1	0	1
3.7	0	3.2	0	4.2	0	3.6	1.8	v
3.1	1	3.5	2	3.5	0	3.0	0	1
2.9	1	3.1	1.2	3.6	1.2	3.6	0	v
2.5	1	3.0	1.1	3.8	0	3.1	1.4	v
3.7	2	3.8	1	3.6	1.3	3.8	1	9
3.8	1.3	3.6	1	3.5	2	3.7	1	9
3.2	1.8	3.7	0	3.6	1	4.0	0	9
2.9	1	2.8	0	3.5	1.1	3.4	1	1

Appendix 6 continued.

	3.1	1	3.3	0	3.6	0	3.2	1.9	1
	3.1	2	3.6	0	3.6	0	3.9	0	1
	2.8	0	3.9	0	3.5	2	3.8	0	1
	3.9	0	3.7	1	3.9	1	3.7	0	1
	2.6	1.1	3.8	1	3.4	1	3.8	0	1
	2.6	1	3.5	0	3.0	0	3.6	0	1
	2.7	1	3.2	0	3.2	1	3.9	1.3	1
Mean	2.9	0.7	3.5	0.6	3.7	0.6	3.6	0.6	
sd	0.4	0.7	0.3	0.6	0.4	0.7	0.3	0.7	

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<sup>a</sup>: see Figure 2.

<sup>b</sup>: N = North; E = East; S = South; W = West.

c = calm sea (0 m waves); m = moderate sea (0.5-1 m waves); r = rough sea



Appendix 6. Viable bacteria in parallel seawater samples taken  
between 1 August and 30 September 1985  
(log<sub>10</sub> units mL<sup>-1</sup>)

.....Site <sup>a</sup> .....								
Ocean		Settling tank		Header tank		Larvae tanks		Prevailing Weather <sup>b</sup>
SWAV	TCBS	SWAV	TCBS	SWAV	TCBS	SWAV	TCBS	
2.8	1	3.5	0	3.9	0	3.8	1	SE, c
2.2	0	3.2	0	3.7	0	3.6	1.2	S, m
2.4	1.5	3.6	0	3.5	1.2	3.5	0	SE, m
2.7	1.3	3.8	0	3.3	0	3.9	0	NW, m
2.9	0	3.2	0	3.4	0	3.1	1	NW, c
3.0	0	3.1	1	3.6	0	3.8	1	N, c
2.9	0	3.8	0	3.1	0	4.0	0	NW, c
2.9	0	3.4	0	3.5	0	3.5	0	W, c
2.8	1	3.6	1.2	3.7	1	3.6	0	W, c
2.7	1	3.6	1	4.2	1	3.1	1.5	SW, c
2.8	0	3.0	1	4.3	2	3.3	1.3	SW, m
2.9	0	3.4	0	3.7	0	3.6	1.2	S, m

Appendix 6 continued.

2.7	0	3.5	1.3	4.0	1	3.7	0	NW, m
3.0	0	3.2	1.5	3.6	0	3.0	1	N, c
3.1	1	3.6	1	4.0	0	3.5	0	E, c
2.8	0	3.7	1	3.5	1.5	3.7	0	E, m
3.1	0	2.9	0	4.8	0	3.4	1	SE, m
2.7	1.5	3.6	1	4.5	0	3.9	2	SE, r
2.6	0	3.7	1	4.0	1.4	3.8	0	S, r
2.8	0	3.4	1.4	4.5	1	3.5	1.2	N, m
3.8	0	3.9	0	3.7	0	4.1	0	NW, m
3.7	0	3.2	0	4.2	0	3.6	1.8	W, c
3.1	1	3.5	2	3.5	0	3.0	0	NW, c
2.9	1	3.1	1.2	3.6	1.2	3.6	0	W, c
2.5	1	3.0	1.1	3.8	0	3.1	1.4	W, c
3.7	2	3.8	1	3.6	1.3	3.8	1	SW, c
3.8	1.3	3.6	1	3.5	2	3.7	1	SW, c
3.2	1.8	3.7	0	3.6	1	4.0	0	SE, m
2.9	1	2.8	0	3.5	1.1	3.4	1	N, m

Appendix 6 continued.

3.1	1	3.3	0	3.6	0	3.2	1.9	NW, c
3.1	2	3.6	0	3.6	0	3.9	0	N, c
2.8	0	3.9	0	3.5	2	3.8	0	NW, c
3.9	0	3.7	1	3.9	1	3.7	0	W, c
2.6	1.1	3.8	1	3.4	1	3.8	0	SW, c
2.6	1	3.5	0	3.0	0	3.6	0	N, c
2.7	1	3.2	0	3.2	1	3.9	1.3	NE, m
Mean	2.9	0.7	3.5	0.6	3.7	0.6	3.6	0.6
sd	0.4	0.7	0.3	0.6	0.4	0.7	0.3	0.7

<sup>a</sup>: see Figure 2.

<sup>b</sup>: N = North; E = East; S = South; W = West.

c = calm sea (0 m waves); m = moderate sea (0.5-1 m waves); r = rough sea (> 2 m waves).